# DEVELOPMENT AND APPLICATION OF AN ANTIBODY-BASED PROTEIN MICROARRAY TO ASSESS STRESS IN GRIZZLY BEARS (*URSUS ARCTOS*)

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**Abstract**

There is an inherent conflict over land use between humans and wildlife. Human activities can alter habitat, creating pressure on North American large carnivore populations. Traditional wildlife techniques can be slow to show population declines, especially in long lived species with slow reproduction rates and high mortality of young, such as grizzly bears (*Ursus arctos*), which leads to delayed information for land managers trying to find the balance between human use of land and preservation of wildlife. Concern about population health of grizzlies in Western Alberta, Canada has lead to investigation of the impacts of current land use within grizzly bear habitat. The objective of this work was to develop a protein microarray that could detect patterns of physiological stress in a rapid manner with small samples of grizzly bear tissue. Sampling from four regions in the foothills of the Rocky Mountains in Alberta resulted in the capture of 133 bears. During the developmental phase, proteins involved with mitochondrial function were found, using two dimensional gel electrophoresis, to be altered in situations of increased stress. Limited cross-reactivity was found when evaluating grizzly bear stress protein expression using commercially available protein microarrays. The protein microarray developed in this thesis consists of 31commercial antibodies validated for grizzly bears. These antibodies recognize proteins associated with different aspects of the stress response, including the hypothalamic-pituitary-adrenal axis, apoptosis/cell cycle, cellular stress, and oxidative stress and inflammation. Skin was selected as the tissue for evaluation of protein expression. Strong correlations were found between many of the proteins within functional categories. Model selection for the protein categories revealed variation that corresponded with region, serum markers of stress (total cortisol and hsp60), growth, the density of roads in the habitat and the amount of anthropogenic change in the bear's home range. Regional trends of expression found bears in Swan Hills and bears from North highway 16 having elevated expression of the proteins measured by the microarray. The protein microarray was thus able to detect expression patterns reflecting physiological and environmental markers. The array shows great promise for future use in detection of potential distress in wildlife populations due to alterations of their habitat.

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| | |
|---|---|
| ACC | apoptosis and cell cycle |
| ACTH | adrenocorticotropic hormone |
| AIF | apoptosis inducing factor |
| Akt | protein kinase B |
| Apaf | apoptotic peptidase activating factor |
| APC | antigen presenting cells |
| AVP | arginine vasopressin |
| ASK | apoptosis signal-regulated kinase |
| BCI | body condition index |
| CCR5 | chemokine (C-C motif) receptor |
| COSEWIC | Committee on the Status of Endangered Wildlife in Canada |
| COX | cyclooxygenase |
| CRH | corticotropin-releasing hormone |
| CS | cellular stress |
| CV | coefficient of variation |
| Cy | cyanine |
| D | dimensional |
| E-Cadherin | epithelial-cadherin |
| eNOS | endothelial nitric oxide synthase |
| FRI | Foothills Research Institute |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| GGT | gamma-glutamyltransferase |
| GR | glucocorticoid receptor |
| GRP | glucose regulated protein |
| HO | heme oxygenase |
| HPA | hypothalamic-pituitary-adrenal axis |

| | |
|---|---|
| HSC | heat shock cognate |
| HSP | heat shock protein |
| IFN | interferon |
| IL | interleukin |
| iNOS | inducible nitric oxide synthase |
| IUCN | International Union for Conservation of Nature |
| JNK | c-Jun N-terminal kinase |
| K | keratin |
| L | leukocyte |
| LPS | lipopolysaccharide |
| MHC | major histocompatibility complex |
| NF | nuclear factor |
| NO | nitric oxide |
| OSI | oxidative stress and inflammation |
| PAGE | polyacrylamide gel electrophoresis |
| PC | principal components |
| POMC | proopiomelanocortin |
| PRDX | peroxiredoxin |
| R | receptor |
| RSF | resource selection function |
| SOD | superoxide dismutase |
| Th | T helper |
| TNFα | tumor necrosis factor alpha |

# Chapter 1 Introduction

## 1.1 Wildlife population health

There is concern about wildlife population health and sustainability worldwide (Cardillo et al. 2004, 2006, Millennium Ecosystem Assessment 2005). A wildlife population is considered threatened if it meets one of five of the International Union for Conservation of Nature (IUCN) Red List criteria. The IUCN criteria for labeling a wildlife population as either threatened, critically endangered, or endangered and vulnerable, include declining population, geographic range fragmentation or decline, small population size combined with fragmentation or decline, very small population size, very restricted distribution or a high extinction risk (IUCN 2010). Population size is measured as the number of mature individuals capable of reproduction (IUCN 2001). Climate change, harvest and invasive species are some examples of potential threats to wildlife populations (Cardillo et al. 2004, Millennium Ecosystem Assessment 2005, Carroll 2007). Expanding human populations and associated impacts such as habitat loss have also been attributed as causes of wildlife population declines (Cardillo et al. 2004, Millennium Ecosystem Assessment 2005). An integral part of anthropogenic landscape change is road development. Roads have been found to negatively impact some wildlife species through subdivision of populations, direct mortality, avoidance or attraction (Forman and Alexander 1998). Recreational use of wildlife habitat has also been associated with declines in population density of wildlife. Nature-based tourism and recreational use of wildlife habitat are forecast to increase worldwide (Reed and Merenlender 2008). Extinction risk varies between wildlife species. Extinction risks of smaller mammals have been found to be primarily environmental, while in larger mammals (above 3 kg in mass) extinction risk is influenced by environmental factors and intrinsic traits, such as reproductive rate, population density, geographic range size and weaning age (Cardillo et al. 2004, 2005). With increasing anthropogenic landscape change declines in populations are predicted to be more rapid for larger mammalian species (Cardillo et al. 2005). Conservation of wildlife species requires understanding of the biogeographic patterns, community structure, population dynamics, and individual behaviour and health (Deem et al. 2001). Biogeography is defined as where a species lives, at what abundances and why they are

present or absent in a particular geographic area (Martiny et al. 2006). The individual health issues of interest are those that could affect population health (Deem et al. 2001).

There are numerous field techniques available to monitor population health, but many are labour intensive and expensive. These field techniques include monitoring trends in abundance, mortality and reproductive rates. Monitoring population numbers, births and mortalities are techniques slow to produce information on population health, and are relatively insensitive to immediate changes within populations (Mattson et al. 1996). Conservation physiology, monitoring physiological responses of organisms to human habitat alteration that may cause or contribute to population declines, is another, potentially more expedient way to evaluate populations that has been employed in the field in recent years (Wikelski and Cooke 2006). Studies of endocrinology, immunology, epidemiology and physiological genomics are some examples of disciplines that are encompassed by conservation physiology (Wikelski and Cooke 2006). Some of the tools of conservation physiologists can even be employed remotely with minimal disruption to the animal (Wikelski and Cooke 2006). Conservation physiology is a developing field which can encompass many sensitive and reliable techniques to assess wildlife population health. Connecting physiological changes to anthropogenic activities, such as habitat alteration, will be important for demonstrating that the physiological changes observed are not within the normal fluctuations of the species. Gene expression changes have been linked to reproductive changes in a population after exposure to a chemical stressor (Heckmann et al. 2008). The capacity to anticipate problems early enough to take corrective action is vital for ecological forecasting, such as wildlife population sustainability analysis (Clark et al. 2001).

Protein microarrays are one of the latest methods for monitoring human health in the clinical setting (Barrier and Mirkes 2005, Cahill 2001, Cutler 2003, Espina et al. 2003, Haab 2003, Liotta et al. 2003, Templin et al. 2003, Walter et al. 2002). Similar to technology used in gene (cDNA) microarrays, protein arrays consist of a series of molecules that are immobilized (spotted) onto known locations on a matrix, allowing determination of increased or decreased protein expression compared to a reference sample. In human studies, patterns of protein expression detected by protein microarray have been correlated with pathological states (Wilson and Nock 2003). Protein arrays are able to determine the protein expression patterns in tissue homogenates or serum. The need for protein arrays stems from the generally poor correlation

observed between mRNA and protein expression in cells (Cutler 2003). Advantages of protein microarrays include speed, sensitivity and the ability to use small samples, which is very beneficial when monitoring wildlife in a non-lethal manner (Wilson and Nock 2003).

### 1.2 Stress

Stress can be defined as a state of threatened homeostasis caused by physiological, psychological or environmental stressors (Black and Garbutt 2002). One of the pioneers of the study of stress was Walter Cannon, who proposed that organisms had an evolutionarily adaptive physiological reaction to acute stressors, involving adrenalin and the mobilization of energy (Cannon 1914). Another pioneer in stress research was Hans Selye who studied organism reactions to stress and proposed the 3-step general adaptation syndrome (alarm-resistance-exhaustion) (Selye 1936). The stress response results in the heightening of the senses, diminished sensitivity to pain, mobilization of energy and disruption of various biological processes (digestion, reproduction, growth, immune response) (Boonstra et al. 1998, Curry and Edwards 1998). An animal's perception of a stressor, shaped by physical condition, physiology and prior exposure to the stressor, can influence the nature and extent of the stress response (Curry and Edwards 1998, Curry 1999, Romero 2004). An animal's reaction to a stressor is also affected by the type, intensity and duration of the stressor (Curry 1999). Stressors that represent immediate threats to physiological homeostasis activate somatic, visceral or circumventricular sensory pathways. Stressors that an animal anticipates, using higher cortical processes, activate limbic-sensitive pathways (Tilbrook and Clarke 2006). Short term stress can be considered adaptive, such as avoiding predators, dealing with overheating resulting from summer weather, exercise, or immunological challenge. Long-term stress on the other hand, is generally maladaptive and can have various deleterious effects on an individual's health, long-term survival and reproductive output (Boonstra et al. 1998). Chronic exposure to physiological mediators outside normal operating ranges, due to long-term stress, can lead to physiological dysregulation of interrelated physiological systems over time. Physiological dysregulation can lead to poor psychological, physical and cognitive functioning and could be viewed as an early indicator of morbidity and mortality (Seplaki et al. 2005).

Long-term stress can have population as well as individual level effects. Chronically elevated glucocorticoid levels have been found to suppress growth, inhibit reproduction,

suppress the immune system, affect thyroid function, disrupt secondary cell messengers, disrupt learning and memory, suppress growth and cause neuronal cell malfunction (Charmandari et al. 2005, Das et al. 2005, Wingfield 2005). Stress can be a risk factor for disease, since prolonged stress has been found to accelerate disease processes. The risk for developing disease in an individual is an interaction of genetics and environmental factors (Schmidt et al. 2008). Long-term stress can also have metabolic effects, as glucocorticoids suppress the actions of growth hormone and sex steroids on fat tissue, muscle and bone, which can result in increased visceral adiposity and decreased muscle and bone mass (Charmandari et al. 2005). Corticotropin-releasing hormone (CRH) can also decrease gastric acid secretion and emptying which affects colonic motor function. Individual heterozygosity in stress responsiveness may result from genetic polymorphisms in CRH and arginine vasopressin (AVP) receptors or regulators (Charmandari et al. 2005). Extrapolation to population health from the measurements performed on individuals is a key goal when studying wildlife. Impairments that interfere with or modulate an individual's normal responses to changes in environmental factors, such as nutrition, infections or climatic conditions, may negatively affect the long-term persistence of populations, through changes in mortality or reproduction (Deem et al. 2001, Wobeser 2006). A major concern is the potential effects of long-term stress on individual health (growth, reproduction and immune function) resulting in negative effects on population dynamics.

### 1.2.1 Allostasis

Animals in any environment have predictable changes to deal with, such as breeding and hibernating, and unpredictable changes encountered, such as storms. The process of maintaining stability through change as a way an organism deals with unpredictable or predictable challenges is termed allostasis (McEwen and Wingfield 2003). Allostasis is a continuum of all predictable and unpredictable events that includes the effects of experience, body condition and habitat. The cumulative cost of allostasis to the body is termed allostatic load. Allostatic load is the toll on the body resulting from its attempts to adapt to physical or psychosocial challenges (McEwen 2000). Allostatic load can result from long-term stress (frequent stimulation of the physiological stress response by novel stressors), repeated exposure to the same stressful event to which the body fails to habituate, failure to terminate the stress response efficiently and inadequate hormonal stress response which allows excessive activity of other allostatic systems, such as the

inflammatory response of the immune system (McEwen 1998, 2000). Individual differences in response to stressful challenges result from perception and interpretation of the situation, body condition, and genetics; this in turn influences an individual's allostatic load (McEwen 1998, McEwen and Seeman 1999).

Allostatic overload is the condition where an animal's ability to cope is overwhelmed and the potential for harm is high (McEwen and Wingfield 2003). Allostatic overload has been broken down into 2 categories: Type 1 occurs when energy supply cannot meet energy demand and Type 2 occurs when there is adequate energy consumption accompanied by social stressors. Type 1 allostatic overload triggers temporary physiological changes, an emergency life stage, that allows an animal to survive. The emergency life stage lowers energy demand by sacrificing energetic input into processes such as growth or reproduction. If the energy supply is now sufficient to meet demand, the Type 1 overload ends and the animal's stress response is lowered (McEwen and Wingfield 2003, Wingfield 2005). Emergency life stage is adaptive if it lasts for a short time, inhibiting reproductive behaviour, regulating the immune system, increasing gluconeogenesis, and increasing foraging behaviour. If the emergency life stage is prolonged, detrimental effects occur such as inhibited reproduction, suppressed immunity, breakdown of skeletal muscle and reduced growth (Wingfield and Romero 2001). Type 2 allostatic overload has been observed in humans and captive mammals and is caused by social conflict, captivity, injury, depression and a lack of control. Type 2 allostatic overload does not trigger an emergency life stage and can only by alleviated through learning or changes in the environmental conditions, such as social structure (McEwen and Wingfield 2003, Wingfield 2005). Fear and anticipatory anxiety can also affect energetic balance as the behavioural changes associated with fear can change access to food resources, for example a dominant conspecific can influence where, when and the duration of foraging (McEwen and Wingfield 2010). Beyond energetic demands of allostatic load there is also the issue of sustained elevation and dysregulation of stress mediators, which can contribute to allostatic load (McEwen and Wingfield 2010). There is concern that the frequency, duration and intensity of changes that free-living vertebrates face in their environment has increased because of human activity, such as logging and urban development (Wingfield 2005, McEwen and Wingfield 2010).

Within the realm of human medicine a cumulative index of biological health risk has been created, termed allostatic load (McEwen 1998, McEwen and Seeman 1999). Symptoms of allostatic load, in humans suffering from anxiety disorders, hostile and aggressive states, depression and post-traumatic stress disorder, include chemical imbalances, perturbations of diurnal rhythm and in some people atrophy of regions of the brain (McEwen 2000). There is evidence of an association between cardiovascular disease, other systemic disorders, and depression and hostility (McEwen 2000). In these human studies, allostatic load was evaluated by scoring individual indicators of physiological activity relating to major regulatory systems, such as the hypothalamic-pituitary-adrenal (HPA) axis, metabolic processes and the cardiovascular system. Individuals with scores in the top quartile across all patients for individual health measures were assigned a point. The allostatic load score was the sum of all the points for each patient (McEwen 2000, Seeman et al. 2004). The allostatic load score is postulated to reflect the diurnal rhythms of stress mediators, diet as well as stress (McEwen 2000). Allostatic load was found to account for more of the variance between patients than individual health measures, and had higher predictive value for morbidity and mortality than individual physiological markers (Seeman et al. 1997, Seeman et al. 2004). The allostatic load during childhood associated with poverty has been observed to predict working memory in young adulthood (Evans and Schamberg 2009). An association has been observed between lower socioeconomic status and higher allostatic load (Bird et al. 2009, Seeman et al. 2010). Higher allostatic load scores have been associated with declining cognitive and physical function in elderly patients as well as being associated with increased risk of cardiovascular disease, abdominal obesity, hypertension, diabetes and arthritis (Seeman et al. 1997, Mattei et al. 2010).

### 1.2.2 Reactive scope model

The reactive scope model is an alternative proposal to the allostatic load model (Romero et al. 2009). Reactive scope has four ranges for concentrations of physiological mediators involved in the stress response. Normal reactive scope of an organism includes predictive homeostasis, the range that encompass circadian and seasonal variation, and reactive homeostasis, the range that responds to unpredictable or threatening environmental changes. Reactive homeostasis is equivalent to the emergency life history stage described above. Homeostatic overload occurs when the concentrations of mediators rise above the normal

reactive scope, while homeostatic failure occurs when levels fall below normal reactive scope. Homeostatic overload will be detrimental to individual health if persistent, which may manifest as pathology and disease. Homeostatic failure could be detrimental more acutely. Cost or wear and tear of maintaining physiological mediators in the reactive homeostasis range is modeled by a decrease in threshold between reactive homeostasis and homeostatic overload. The reactive scope model tries to bypass the perceived weaknesses of the allostatic model, such as a dependence on energy use which can fluctuate with stressors, different life history stages and interspecific differences. Mediators included in the reactive scope model include immune, HPA, cardiovascular and central nervous system mediators as well as behavioural changes. Different mediators are modeled separately. This model can also accommodate responses in anticipation of a stressor as well as a response to an actual stressor. The reactive scope model slopes can be determined empirically with young, healthy, naïve animals and are expected to differ between species (Romero et al. 2009). Direct applications of this empirical determination may prove problematic for wildlife species, but the principals could be applied.

### 1.3 Monitoring wildlife health

Within the arena of wildlife research there is concern that the stress wildlife experience has been increasing with increasing anthropogenic activity in their habitats and landscape modification, and that increased stress on individuals may impact population health of certain species (Wasser et al. 1997, Wasser et al. 2004, Martinez-Mota et al. 2007, Macbeth et al. 2010). As well as the potential for detrimental effects of long-term stress, there is concern that the energetic demand of allostasis in addition to the energetic demands of life history stages such as breeding, molting, migration and hibernation may create conditions of negative energy balance which can result in the switch to an emergency life stage during which reproduction and growth of the organism are suppressed (Arlettaz et al. 2007, McEwen and Wingfield 2003).

Collecting a large tissue specimen from a species in the wild could lead to infection and cause further stress to an already potentially stressed individual. Like other field methods, in wildlife biology the goal is not to do harm to the animals. However, protein samples obtained from small skin and muscle tissue biopsies could be collected from bears without causing undue stress. From these small tissue samples, patterns of stress-associated proteins determined by an increase or decrease of protein expression using a protein microarray could be analyzed and

validated by correlation to other field data, such as body weight, immune markers in the blood, reproductive status or history, and landscape of the animals. A protein microarray of this type may provide an essential tool for early warning of problems in bear populations that would indicate the need for further attention. Furthermore, if the protein microarray is based on evolutionarily conserved stress proteins, it potentially could be applied to a wide range of wildlife species of concern.

Thus, protein microarrays could be used to evaluate a suite of stress proteins in free-ranging wildlife. This suite of proteins could represent various stress-associated processes in the animal, such as oxidative stress, cellular proteotoxicity, neuroendocrine regulation, and cell cycle control (i.e., apoptosis and mitosis). Use of this technique may therefore provide unique and detailed information regarding animal stress. As there is no single consistent biological response to long-term stress, it can be difficult to identify and measure (Curry and Edwards 1998). Mammalian physiology is a complex web of induction and feedback inhibition cascades, and proteins associated with stress may be either induced or suppressed. Depending on the type of stressor some inducible proteins may suppress the expression of other proteins via feedback loops in the stress response (Tsigos and Chrousos 2002). Importantly, both increases and decreases of stress protein expression are detectable using protein microarray technology.

### 1.4 Grizzly bears

Habitat alteration is a concern for the sustainability of wildlife populations. Large mammalian predators with long lives, high mortality of young, a developmental period of years before beginning reproduction, and slow reproduction due to nurturing young for extended periods, are at highest risk because problems at the population level may be difficult or take a long time to detect. In addition, such wildlife populations have a low probability of recovering quickly assuming the cause of the population decline can be determined and management decisions can be made to help the population (Garshelis 2002, Mattson et al. 1996, Pasitschniak-Arts 1993). Grizzly bears (*Ursus arctos*) in certain areas of North America fit into this risk category.

Concerns about the sustainability of grizzly bear populations stem largely from changes in their habitat. Grizzly bears are theorized to have a lower resistance to environmental disturbances because of the strong philopatry of female offspring to maternal home ranges, the

need for quality forage in spring and fall, and their low reproduction rate (Weaver et al. 1996). On the western slopes of the Rocky Mountains, grizzly bear populations that were partially isolated from the continuous bear population to the north were found to have a lower population density. As the food availability of the two areas was not found to be different it was theorized that the difference in density resulted from human impacts on survival and bear habitat (Mowat et al. 2005). Grizzlies have expansive home ranges, from 24 to 1398 $km^2$ for males and from 12 to 430 $km^2$ for females depending on the distribution of food, distribution of cover, topography and various individual characteristics, such as, age, social status and condition of the animal (Pasitschniak-Arts 1993). Habitat alteration is extensive and occurring rapidly in some areas because of resource exploration and development and human recreational activities. In recent years extensive alteration of habitat in western Alberta, Canada due to industrial development, mining, oil and gas extraction, forestry, municipal development, recreation and road construction has occurred. Roads fragment habitats and the negative effects of this have been observed in bears as well as other wildlife. Roads allow greater human access into bear habitat which can lead to human-caused grizzly bear mortality (McLellan et al. 1999, Ciarniello et al. 2007, Nielsen et al. 2008, Graham et al. 2010). It has been reported that grizzly bears underutilize habitat near roads, especially female bears (Mattson et al. 1996, Gibeau et al. 2002). By avoiding roads and traffic noise, grizzly females have been found to avoid certain high quality habitats (Gibeau et al. 2002). Female grizzly bears in west-central Alberta have been found to cross roads more frequently than males, especially in daylight hours (Graham et al. 2010). Females crossing roads would have a greater chance of human encounters, which could increase the incidence of female grizzly bear mortality (Graham et al. 2010). Female grizzly bears were also found more often using areas closer to human settlements and places where people may be encountered such as parks, areas where bears encounter humans and off-road vehicles as well as cars and trucks (Gibeau et al. 2002). The use of sub-optimal habitats and increased probability of encounters with humans could have negative impacts on female survival and reproductive output.

Humans are the leading cause of grizzly bear deaths in the Rocky Mountain area, despite controlled hunts and legal protection (Mattson et al. 1996, Benn and Herrero 2002, Garshelis et al. 2005). It has been estimated in areas of interior Rocky and Columbia mountains with legal hunting >70% grizzly bear mortality is human related, while in areas without legal hunting

where humans have greater access to the habitat the number of bears killed by humans that are unreported is likely similar to the number reported (McLellan et al. 1999). A positive correlation has been found between reduced habitat use by bears, bear death and human access to bear habitat (Mattson et al. 1996, Benn and Herrero 2002, Nielsen et al. 2004b). Bears that become habituated and food-conditioned live more successfully near humans, but they have a higher probability of a shortened lifespan (Mattson et al. 1996, Gibeau et al. 2002). Grizzlies may choose less suitable habitats in order to avoid the stress of human interaction. There is some evidence of adult males using the back-country, less human accessible habitats, forcing adult females and younger bears into areas with high probability of human contact (Mattson et al. 1996). Today it is increasingly difficult to find areas where humans do not have access. Altered bear behaviour, increased bear-human conflicts, and increased need for bear removals and displacement of certain cohorts, such as females with young, have been found to result from increased human access to the backcountry (Benn and Herrero 2002).

### 1.5 Grizzly bears in Canada

In 2002, the status of the grizzly bear in Canada was classified as a species of *Special Concern* by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC). Concern was raised about human use of grizzly bear habitat (Ross 2002). The government of Alberta designated the grizzly bear as a threatened species on June 3, 2010. Use of bear habitat for human recreation and resource extraction were primary concerns (Alberta Sustainable Resource Development 2006). Modeling of the relative risk of mortality of grizzly bears in the central Rocky Mountains of Canada found that risk of mortality was positively associated with human access (Nielsen et al. 2004b). Grizzly bear females from the eastern slopes of the Rockies in Alberta have been found to have a longer interval between litters as well as lower reproductive rates compared to grizzlies in other areas. At this time it has not been determined why these females have lower reproductive output (Garshelis et al. 2005, Stenhouse et al. 2003). Some possible factors that could affect reproduction are chronic stress, disease and low energy uptake. It has been found in other species of mammals that reproductive function can be compromised when the animal undergoes chronic stress, possibly due to habitat alteration or energy use exceeding energy availability (Chrousos and Gold 1992, Schneider 2004). Reproduction is energetically expensive, and if an animal has a reduced caloric intake then their

reproductive potential may decrease as their body suppresses reproductive hormones (Martin et al. 2008).

Some bears may be lured in by "attractive sinks", areas where there is higher habitat quality linked with higher risk of mortality (Nielsen et al. 2004b). It has been predicted that the grizzly bear, an indicator of health of terrestrial ecosystems, will continue to experience habitat alterations and population declines in the Canadian Rockies (Benn and Herrero 2002, Nielsen et al. 2004b), thus threatening the sustainability of certain populations.

One issue for wildlife managers trying to sustain grizzly bear populations is setting sustainable harvest rates. Male biased hunting is supposed to protect grizzly bear populations, but it has been suggested that excessive adult male mortality may allow immigration of subadult and younger adult males that may displace females and kill their cubs (Mattson et al. 1996). A three year moratorium of licensed grizzly bear hunting in Alberta was established in 2006 and extended through 2009 and 2010 (Alberta Grizzly Bear Recovery Team 2008, Government of Alberta 2010). In order to accurately set hunting limits and protect bear populations, there must be information on both the absolute numbers of animals and the health of the bear populations. Resource utilization industries also require this information, in order to determine what habitats are needed to maintain healthy bear populations.

### 1.6 Foothills Research Institute (FRI) grizzly bear research

In 2008, the Alberta grizzly bear recovery plan was released. One of its recommendations is to increase the knowledge base on grizzly bear habitat and health data (Alberta Grizzly Bear Recovery Team 2008). The Foothills Research Institute (FRI) grizzly bear research program brings together collaborators from several academic institutions as well as the Alberta government to gather information on food resource use, habitat structure and use, and grizzly bear health in Alberta (Stenhouse and Graham 2008). This project is just one of several pertaining to Alberta grizzly bear health.

### 1.7 Proteins involved in the stress response

Animals have an evolutionary conserved suite of proteins that help their bodies deal with stress. These proteins respond to multiple stressors, such as infection and contaminants. Patterns of stress protein induction and suppression represent many important physiological

processes, such as apoptosis, mitosis, cellular detoxification, oxidative stress, and neuroendocrine regulation. In the following sections, key proteins involved in various aspects of the stress response will be introduced.

### 1.7.1 Hypothalamic –pituitary-adrenal (HPA) axis

The molecular cascade after perception of a stressor is rapid. Within seconds of stress perception, there is increased secretion of epinephrine and norepinephrine from the sympathetic nervous system and adrenal medulla, increased secretion of oxytocin from the neural lobe of pituitary and release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from pericellular neurons of the hypothalamus into the portal circulation (Carrasco and Van de Kar 2003). AVP potentiates the stimulatory effects of CRH on adrenocorticotropic hormone (ACTH) secretion (Carrasco and Van de Kar 2003, Volpi et al. 2004). Release of CRH and AVP from pericellular neurons into the hypophyseal portal system and delivery to the anterior pituitary initiates endocrine release of proopiomelanocortin (POMC) derivatives, such as ACTH (Carrasco and Van de Kar 2003, Charmandari et al. 2005). Within five to ten seconds there is an increase in ACTH secretion from the pituitary into the general circulation. Seconds later, there is a decrease in secretion of pituitary gonadotropins and increase in secretion of prolactin, growth hormone, glucagon and renin from the anterior pituitary, pancreas and kidneys, respectively (Carrasco and Van de Kar 2003). Within minutes the adrenal cortex releases glucocorticoids into the general circulation, which produces immediate mobilization of glucose for muscle and stimulation of hepatic gluconeogenesis (Boonstra 2005). Physiological changes resulting from glucocorticoids include increased focus on perceived threats, mobilization of energy for brain and muscle function, redistribution of blood flow, enhanced respiration, heightened cardiovascular output, decreased feeding behaviour, alteration of immune function, and inhibition of reproductive behaviour (Carrasco and Van de Kar 2003). As mentioned previously this is adaptive in the short term, but can cause deleterious effects with repeated or long-term exposure to stress.

One of the first neuropeptides released in the HPA cascade is CRH (also known as corticotropin releasing factor or CRF), which stimulates POMC expression and release of ACTH. Corticotropin-releasing hormone is also a participant in the activation of the sympathetic nervous system (Carrasco and Van de Kar 2003). Two receptors for CRH, CRH-R1 and CRH-

R2, are found in the central nervous system as well as some areas in the periphery including the skin. The predominant form found in the brain and pituitary is CRH-R1, while CRH-R2 is the more prevalent form expressed in peripheral tissues (Slominski et al. 2001). CRH-R1 expression in the hypothalamus is rapidly up-regulated in response to stress and CRH (Herman et al. 2003). Both CRH-R1 and CRH-R2 appear to be reciprocal in their roles in modulating anxiety and stress induced behaviour, with CRH-R1 involved in anxiety-like behaviour and initiating the acute phase of the HPA stress response and CRH-R2 involved in a lessening of anxiety and recovery from stress (Carrasco and Van de Kar 2003). Corticotropin-releasing hormone has been associated with immunomodulation both systematically and locally in the skin (Slominski and Wortsman 2000). Corticotropin-releasing hormone and its receptors are therefore intimately involved in the body's reaction to stress.

Secreted from the hypothalamus, AVP acts synergistically with CRH to activate secretion of ACTH and is itself a weak activator of ACTH secretion (Carrasco and Van de Kar 2003). Implicated as a neuropeptide that allows for bypass of the negative glucocorticoid feedback, AVP may allow for further reaction to stressors in an animal already reacting to a stressor. It has been suggested that in cases of repeated activation of the HPA axis, AVP may be able to override the negative glucocorticoid feedback of ACTH release, resulting in continued responsiveness of corticotrophs to novel stressors (Carrasco and Van de Kar 2003). With chronic stress an increase in AVP expression and secretion was found (Black 2002, Carrasco and Van de Kar 2003, Volpi et al. 2004, Schmidt et al. 2008). Differences in reaction to specific stressors exist also between the sexes. When challenged with a psychological stressor males employ AVP as the primary hormone to cope, whereas oxytocin is the primary hormone used in females (de Kloet 2003). Genetic polymorphisms in CRH and AVP receptors or regulators have been theorized to account for individual heterozygosity in stress responsiveness (Charmandari et al. 2005). In addition to ACTH release, AVP is involved in body fluid homeostasis, vasoconstriction and facilitating social and reproductive behaviours (Donaldson and Young 2008, Aoyagi et al. 2009). AVP V1a receptor, one of the subtypes of AVP receptors, is widely distributed having been located in brain, platelets, blood vessels, liver, adrenal gland and uterus (Carrasco and Van de Kar 2003).

Proopiomelanocortin is released from the anterior pituitary following stimulation by CRH and AVP. Prohormone convertases process POMC into ACTH, β-endorphin and α-melanocyte

stimulating hormone. Adrenocorticotropic hormone is a key regulator of glucocorticoid secretion from the zona fasciculata in the adrenal cortex (Carrasco and Van de Kar 2003, Charmandari et al. 2005). Adrenal androgens and aldosterone are also regulated by ACTH (Charmandari et al. 2005). Negative feedback acts to limit the duration of the stress reaction by inhibiting POMC gene expression (Sapolsky et al. 2000, Slominski et al. 2000, Dostert and Heinzel 2004).

Glucocorticoids are involved in basic physiological functioning as well as the stress response. Glucocorticoids are involved in carbohydrate and lipid homeostasis, immune function regulation, negative feedback on the HPA axis, and gene regulation. Baseline glucocorticoid levels have been found to regulate immune function, increase gluconeogenesis and fat storage, regulate ion transport and provide negative feedback for release of ACTH and CRH (Wingfield 2005). Basal glucocorticoid levels change temporally as do responses to stressors (Boonstra 2005). Glucocorticoids have two types of receptors, high affinity mineralocorticoid and lower affinity glucocorticoid receptors (GR) (Carrasco and Van de Kar 2003). Mineralocorticoid receptors are responsible for selection of behavioural responses to stress, controlling the sensitivity of the stress system and preventing disruption of cellular homeostasis. Glucocorticoid receptors are involved in behavioural adaptation, facilitating recovery of cellular homeostasis, promoting memory formation, controlling energy metabolism, and restraining stress-induced responses (de Kloet 2003). The number of GRs is highly variable depending on cell type (Sapolsky et al. 2000). After activation the receptor translocates to the nucleus where it binds to glucocorticoid response elements located in the promoter region of target genes. Binding of glucocorticoid response elements can result in either negative or positive regulation depending on the gene. Activated glucocorticoid receptors can also physically interact with transcription factors to modulate gene expression (Charmandari et al. 2005). Glucocorticoids can down-regulate GRs (Sapolsky et al. 2000). Medical students undergoing examination stress were found to have decreased levels of GR (Webster Marketon and Glaser 2008). Chronic stress reduced mineralocorticoid receptor and GR mRNA levels in rat hippocampus (Chao et al. 1993, Sterlemann et al. 2008). Central GR desensitization to the negative feedback inhibition by HPA axis has been found with chronic stress (Leonard 2005).

Prolactin has physiological roles beyond its well known involvement in pregnancy and lactation. Prolactin is involved in immune regulation, growth and osmoregulation of epithelial tissues. Prolactin has been found to be immunostimulatory (Paus 1991, Webster Marketon and Glaser 2008). Prolactin is released following exposure to various stressors, such as competitive stress, occupational stress and exercise (Mastorakos et al. 2005, Aizawa et al. 2006, Tomei et al. 2006). Other stressors, such as hostile behaviour, parental stress and energetic constraints have been found to result in decreased prolactin levels (Cherel et al. 1994, Malarkey et al. 1994, Angelier and Chastel 2009). Prolactin has been found to reduce HPA responsiveness to stress in both males and females (Tilbrook and Clarke 2006). Glucocorticoids can inhibit prolactin gene expression (Dostert and Heinzel 2004, Sapolsky et al. 2000). Prolactin is secreted from the anterior pituitary as well as extra-pituitary sites, such as peripheral blood lymphocytes, skin, and hair follicles (Paus 1991, Arck et al. 2006, Webster Marketon and Glaser 2008).

The skin has been found to have a peripheral equivalent of stress-activated HPA axis (Arck et al. 2006, Hosoi 2006, Paus et al. 2006). Skin, the body's largest organ, is responsible for maintaining homeostasis while exposed to a changing external environment. Mammalian skin produces POMC, ACTH, glucocorticoids and GR (Slominski et al. 2000, Slominski and Wortsman 2000, Ito et al. 2005, Arck et al. 2006). Prolactin is produced by mouse skin as well as mouse and human hair follicles (Arck et al. 2006). Species differences have been found, with human skin producing CRH, while mouse skin may receive CRH from a neuronal source (Ito et al. 2005). In human and mouse skin, CRH-R1 and CRH-R2 have been detected with species specific localization (Slominski et al. 2007). In mice, CRH-R2 was found in all skin components tested (Slominski et al. 2004). Although the enzymes responsible for processing POMC to ACTH and other products have been detected in human skin, epidermal keratinocytes and melanocytes, cutaneous nerves, and circulating immune system factors may contribute POMC peptides to the skin (Slominski et al. 2000, Konig et al. 2006). In mouse skin, POMC expression and production of POMC peptides is inhibited by glucocorticoids and affected by the hair growth cycle (highest levels found in anagen/growth phase) (Slominski et al. 2000, 2007). Skin expression of CRH, POMC and ACTH are also affected by interleukin (IL) 1 and tumor necrosis factor α (TNFα) release, cutaneous pathology, and ultraviolet radiation (Slominski et al. 2000). Skin pigmentation, immune response and auxiliary systems can be regulated by CRH and POMC peptides (Arck et al. 2006). Acute stress has been found to increase skin CRH expression and

enhance skin immune function while long-term stress suppresses skin immunity (Dhabhar 2000, Ito et al. 2005). Acute stress enhancement of immunity is thought to prepare the skin for potential wounding and risk of infection (Dhabhar 2000). Hair follicles have also been found to produce CRH, CRH receptor, GR, glucocorticoids and POMC (Slominski and Wortsman 2000, Slominski et al. 2000, Kono et al. 2001). Melanocytes have been found to produce glucocorticoids (Arck et al. 2006). In hair follicles, increasing CRH has been reported to up-regulate CRH-R1 and 2, and ACTH stimulates the production of glucocorticoids; in addition there is evidence of the presence of negative feedback control of glucocorticoid production in skin (Ito et al. 2005). Increased glucocorticoids, resulting from psychological stress, impair skin integrity and increase severity of infections (Slominski et al. 2008). Since skin of mammals appears to possess a functional equivalent of the HPA, determination of HPA-associated proteins in skin may be a biologically relevant approach to assess chronic stress in wildlife such as grizzly bears. Central stress axis responses and skin stress responses have been found to be linked (Pavlovic et al. 2008). Psychoemotional stress has been found to increase the number of cutaneous nerve fibers containing substance P, shifts the cytokine profile in the skin toward TH2 and increases the parameters of apoptotic dermatitis (Pavlovic et al. 2008, Evers et al. 2010).

### 1.7.2 Apoptosis and cell cycle proteins

Physiological cell death (apoptosis) is part of normal development and maintenance of healthy tissue. Apoptosis, triggered by pro-death signals, is a highly regulated cell death cascade characterized by cytoplasmic shrinkage, nuclear disintegration, DNA fragmentation, membrane blebbing, and finally fragmentation of the cell into apoptotic bodies (Degterev et al. 2003). Apoptosis is an important route for clearing dysfunctional cells such as virally infected cells from the body (Cohen 1997). There are two main apoptosis pathways in mammalian cells. In the mitochondrial pathway, mitochondria play a key role by initiating the release of cytochrome c, which results in assembly of the apoptosome and subsequent downstream activation of caspase 9 and caspase 3. In the death receptor pathway, binding of members of the death-receptor family, Fas and TNFα, to their receptors results in formation of an apoptosis inducing signaling complex and subsequently activation of caspase 8 and caspase 3 (Hengartner 2000, van Empel et al. 2009). Diseases such as neurodegeneration, autoimmunity and cancer can result from dysregulation of apoptosis (Degterev et al. 2003).

The mitochondrial pathway of apoptosis is mediated by the mitochondrial flavoprotein apoptosis-inducing factor (AIF). Induction of the mitochondrial pathway of apoptosis results in AIF translocating from the mitochondrial intermembrane space to the nucleus where it facilitates chromatin condensation and DNA fragmentation (van Empel et al. 2009). Apoptosis-inducing factor may have oxidoreductase and peroxide scavenging activities and therefore may also have an anti-apoptotic role by regulating the production of reactive oxygen species (Krantic et al. 2007). Protection against neuronal apoptosis caused by oxidative stress was found to be provided by AIF (van Empel et al. 2009).

Caspases are cysteinyl aspartate-specific proteases that play a central role in apoptosis (Lamkanfi et al. 2007). Caspases are selective about which proteins they cleave (Hengartner 2000). Caspases are often categorized as initiator or executioner caspases. Initiator caspases function at the beginning (upstream portion) of the apoptosis cascade, while executioner caspases are involved downstream in the apoptosis cascade. Caspase 1 is considered part of the inflammatory caspase group as it is involved in both inflammation and apoptosis (Degterev et al. 2003, Lamkanfi et al. 2007). Caspase 1 is an initiator caspase, however it is has not been found to be necessary for apoptosis (Cohen 1997). Caspase 1 is also responsible for the maturation of pro-IL 1β and pro-IL18 (Lamkanfi et al. 2007). Caspase 1 is activated in response to a number of bacterial ligands and nucleic acids (Lamkanfi et al. 2002, Lamkanfi et al. 2007). Caspase 2, which has properties of both an initiator and effector caspase, is one of the best conserved caspases across species. Apoptosis triggered by DNA damage, TNF, and different pathogens and viruses appears to need caspase 2 at the onset. Two isoforms of caspase 2 exist, 2L and 2S. Caspase 2 is thought to play a role in both positive and negative regulation of cell death. Overexpression of caspase 2L and 2S result in induction and suppression of cell death, respectively. Caspase 2 appears to engage the mitochondrial apoptotic pathway by permeabilizing the outer mitochondrial membrane and or by changing the association of cytochrome c with the inner mitochondrial membrane (Degterev et al. 2003, Zhivotovsky and Orrenius 2005). Caspase 6 is an executioner caspase (Degterev et al. 2003). Although apoptosis in most cases does not require new protein synthesis, in certain circumstances such as lipopolysaccharide (LPS) exposure or ischemia, caspase transcription is upregulated (Degterev et al. 2003).

Proteins within the annexin family are involved in a diverse array of functions within the cell from plasma membrane dynamics to cellular differentiation. Annexins have been linked to the cell cycle (Schlaepfer and Haigler 1990). Annexins are highly conserved calcium effector proteins, expressed in a variety of tissues (Gerke and Moss 2002). Dramatic changes in calcium and pH homeostasis result from the activation of intracellular pathways via chronic physiological stress. Annexins respond to disease and stress-related cellular changes to assist in the restoration of intracellular homeostasis (Monastyrskaya et al. 2009). Annexin II has been implicated in wound closure (Monastyrskaya et al. 2009), and has been linked to stabilization or formation of actin assembly and attachment sites at cellular membranes (Gerke et al. 2005). The ability of annexin II to organize ordered lipid microdomains might be important for stabilization of cell-to-cell contact sites in endothelial and epithelial monolayers and in cell signaling. Annexin II in smooth muscle may be involved in muscle contraction (Gerke and Moss 2002, Gerke et al. 2005). Annexin II has been found to be induced in certain cells by changes in cellular redox state, both hypoxia and hyperoxidative stress (Gerke and Moss 2002). Both annexin II and IV have been hypothesized to be involved in endocytosis and exocytosis. Annexin IV has been found to have an inhibitory effect on $Ca^{2+}$-dependent $Cl^-$ ion channel activity in epithelial cells (Monastyrskaya et al. 2009). The activity of NF-κB, a transcription factor that regulates genes involved in immune response, cell survival and apoptosis, is affected by annexin IV in a $Ca^{2+}$ dependent manner. Annexin IV may be a substrate for caspase 3 (Jeon et al. 2010).

Epithelial (E)-cadherin is involved in maintaining structural integrity of the tissues in which it is expressed as well as assisting cellular communication and antigen processing. E-cadherin is expressed on cell surfaces of all epidermal layers and is involved in selective adhesion of epidermal cells (Furukawa et al. 1997). E-cadherin is involved in the immune response by affecting the persistence in the epidermis of Langerhans cells, which present antigens to T helper cells (Tang et al 1993). Actin cytoskeleton-regulated cell communication in various developmental and pathological conditions has been found to depend on E-cadherin-mediated adherens-type intercellular junctions (Mege et al. 2006).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is involved in many cellular processes, such as endocytosis, microtubule bundling, and apoptosis, and may be involved in RNA and DNA movement and repair. Present in the cytoplasm, nucleus, endoplasmic reticulum,

mitochondria and plasma membrane, GAPDH is a glycolytic enzyme (Tarze et al 2007). GAPDH binds to microtubules, affects their bundling, contributes to membrane fusion and is involved in endocytosis (Hara et al. 2006, Sirover 1997). GAPDH may also be involved with tRNA export, gene transcription, DNA replication, DNA repair and RNA export (Hara et al. 2006, Tarze et al 2007). GAPDH is involved with the initiation of apoptosis and may serve as an intracellular sensor of oxidative stress (Berry and Boulton 2000, Chuang et al. 2005). During apoptosis GAPDH is over-expressed and was found to accumulate in the nucleus and mitochondria (Chuang et al. 2005, Tarze et al. 2007). Within the mitochondria GAPDH is involved in permeability transition pore complex dependent permeabilization of the inner mitochondrial membrane via association with the voltage-dependent anion channel 1 resulting in release of cytochrome c and AIF, a critical step in the mitochondrial apoptosis pathway (Tarze et al. 2007).

### 1.7.3 Cellular stress proteins

Heat shock proteins (hsp) are involved in a diverse group of cellular functions, including cellular protection, protein folding, transport and degradation, and protein-membrane interactions (Kopecek et al. 2001). Heat shock proteins play a role in cellular protection from stressors such as cytokines, energy depletion, hypoxia, acidosis, reactive oxygen species, reactive nitrogen species, viral infection, thermal damage and ischemia (Kregel 2002). Hsps are expressed in the epidermis, dermis and muscle (Garrido et al. 2001, Liu and Steinacker 2001). Different hsp families are classified based on molecular size with individual hsps differing in cellular locations and functions within the cell (Kopecek et al. 2001). Heat shock protein production requires both transcription and posttranscriptional regulatory steps, for instance, a cell can produce more hsp70 mRNA in response to a stressor, with little hsp70 protein production (Kregel 2002).

Heat shock protein 27 is involved in enzyme protection and cellular structure stabilization. Certain detoxifying enzymes, such as glutathione transferase, are protected against oxidation by hsp27. Phosphorylated hsp27 protects cells from oxidative stress by modulating reactive oxygen species and glutathione levels (Arrigo 2001). Hsp27 stabilizes microfilaments and cytokine signal transduction (Liu and Steinacker 2001). Within smooth muscle, hsp27 is theorized to be involved in actin dynamics and cross-bridge function (Gerthoffer and Gunst 2001).

Hsp70 proteins are highly conserved across species and have diverse protective actions within cells. The hsp70 family includes several proteins that are synthesized in response to different stimuli. Hsp73 or heat shock cognate (Hsc70) is present in unstressed cells at a constant level and has been termed constitutive, while hsp72 or hsp70i is highly inducible in response to a variety of stressors. Stressors that result in hsp70i induction include hyperthermia, reactive oxygen species, nitric oxide and other reactive nitrogen species and viral infection. The hsp70 family is involved in protein folding, refolding misfolded proteins, prevention of protein aggregation, maintenance of structural proteins, degradation of unstable proteins and transitory thermotolerance (Kregel 2002). Hsc70 is involved in unfolding proteins prior to their translocation to the mitochondria and targeting proteins for lysosomal degradation (Gething and Sambrook 1992). Hsp70i is induced in muscle during exercise, with heat production being only one of the factors in induction (Kregel 2002). Hsp70i may also be regulated by proinflammatory cytokines such as TNFα, IL-1α, and IL-6 (Liu and Steinacker 2001).

Cellular compartments have specific hsp proteins. Glucose-regulated protein (Grp) 78, also known as binding protein (BiP), is a calcium-dependent chaperone, which binds to partially folded proteins in the endoplasmic reticulum (ER) preventing their aggregation. Grp78 is one of the proteins involved in triggering the unfolded protein response within the ER which slows protein production until unfolded proteins can be folded or marked for destruction, sustains cytosolic calcium homeostasis, hinders apoptosis by forming complexes with pro-apoptotic molecules, such as caspase 7 and 12, acts as a receptor on the plasma membrane, and plays a role in cellular protein secretion (Gething and Sambrook 1992, Feder and Hofmann 1999, Quinones et al. 2008, Zhang and Zhang 2010). Hsp60, a mitochondrial chaperone, binds unfolded proteins and holds them in unassembled states before they are exported or assembled into protein oligomers. Hsp60 also assists in the folding and assembly of polypeptides translocated into the mitochondria (Gething and Sambrook 1992). Within the mitochondria hsp60 is vital for protein maturation (Welch 1993). Hsp60 also functions to refold proteins and prevent aggregation of denatured proteins (Kregel 2002).

Hsp90 has diverse roles within the cell including aiding the conformational maturation of proteins, cell cycle control and involvement in the nitric oxide production. Hsp90 also has roles in regulation of steroid hormone receptors and protein translocation (Kregel 2002). Hsp90

shows specificity in binding proteins aiding conformational maturation of steroid hormone receptors and signal transducing kinases, such as the death domain kinase receptor-interacting protein (Garrido et al. 2001, Vanden Berghe et al. 2003). Hsp90 is also involved in cell proliferation, cell cycle control and mitochondrial homeostasis (Feder and Hofmann 1999, Wandinger et al. 2008). Hsp90 may be involved in the negative feedback loop regulating cellular nitric oxide (NO) production (Wandinger et al. 2008), and is required for endothelial NO synthase (eNOS) activity. Conversely, hsp90 can modified by NO, inhibiting its activity, which inhibits upregulation of eNOS activity by hsp90 (Wandinger et al. 2008).

Hsp110 and hsp40 function as cofactors or "assistants" to other hsps. Hsp110, found in the cytoplasm and nucleus, has been found to enhance the rate and yield of hsp70 mediated protein refolding (Easton et al. 2000, Dragovic et al. 2006). Hsp110 binds to and prevents aggregation of denatured proteins within cells, but does not assist directly in their refolding. Hsp110 assists hsp70 by binding protein while hsp70 folds the protein. Hsp110 is a nucleotide exchange factor for hsp70 (Shaner and Morano 2007). Hsp40 accelerates ATP hydrolysis which enhances hsp70 activity (Shaner and Morano 2007). Hsp40 also chaperones proteins that target proteins to hsp70 and influences hsp70 subcellular location (Fan et al. 2004).

Stress can induce or result in the induction of a variety of hsps. Heat shock proteins were first discovered by exposing *Drosophila melanogaster* to a sudden increase in temperature (Ritossa 1962). Heat shock proteins from the hsp70 family have been found to be the most sensitive to hyperthermia (Kregel 2002). Acquired transitory thermotolerance has been observed with cells and organism which have been exposed to a prior sublethal heat exposure and has been attributed to induction of hsp20, hsp60, hsp70 and hsp110 families (Sanders 1993). Induction of hsps has been associated with increased tolerance to a variety of stressors beyond hyperthermia (Kregel 2002). As natural fluctuations in environmental temperature can result in induction of hsps, recent thermal history should be considered when using hsps as a biomarker (Sanders 1993). Increased core and tissue temperature resulting from resisting snares or culvert traps or running from vehicles used during darting may also have to be considered when evaluating hsps in wildlife species such as bears (Kregel 2002). Hsp27, hsp40, hsp60, hsp70i and hsp90 are induced by various stressors, while hsc70 is constitutively expressed (Charveron et al. 1995, Scheibel and Buchner 1998, Garrido et al. 2001, Kalmar and Greensmith 2009). Induction of

hsp70 depends on the type of stressor; for example acute electric tail shock, but not acute restraint stress, has been found to increase extracellular hsp70 (Campisi et al. 2003).

Heat shock proteins have also been found to be involved in the regulation of apoptosis. Hsp27 and hsp70 have been found to be antiapoptotic. Hsp60 is reported to be both proapoptotic and antiapoptotic (Garrido et al. 2001, Arya et al. 2007, Chandra et al. 2007). Hsp90 is primarily anti-apoptotic (Arya et al. 2007). Hsp110 can be proapoptotic (Cande et al. 2002). Hsp27 can inhibit apoptosis by interacting with components in the apoptotic signaling pathway involved with caspase activation, e.g. caspase 3 and 9 (Garrido et al. 2001, Concannon et al. 2003). Hsp60, when released from the mitochondria, has been found to promote caspase 3 maturation (Xanthoudakis and Nicholson 2000, Garrido et al. 2001, Chandra et al. 2007). Under certain apoptotic conditions, cytosolic hsp60 appears to increase without mitochondrial release and hsp60 promotes cell survival (Chandra et al. 2007). Hsp60 can bind pro-apoptotic Bax and Bak proteins, thereby inhibiting apoptosis (Arya et al. 2007). The role of hsp60 in apoptosis may depend on tissue type, cell type and the apoptotic inducer (Chandra et al. 2007). Caspase 9 maturation can also be prevented by hsp70 and hsp90 binding Apaf1. Hsp70 inhibits AIF induced chromatin condensation and Jun N-terminal kinase (JNK) activation (Xanthoudakis and Nicholson 2000, Garrido et al. 2001, Chandra et al. 2007). Hsp70 is also thought to be able to interfere with the apoptosis cascade downstream of the activation of caspase 3 (Xanthoudakis and Nicholson 2000). Hsp70 can interfere with the release of cell death factors from the mitochondrial membrane (Arya et al. 2007). Hsp90 can induce nuclear factor (NF)-κB mediated inhibition of apoptosis by interaction with Akt and receptor interacting protein (RIP)-1 kinase (Xanthoudakis and Nicholson 2000, Garrido et al. 2001, Chandra et al. 2007). Hsp90, when in complex with Akt, can inactivate ASK-1, which inhibits JNK-mediated cell death (Arya et al. 2007). In contrast, the mitochondrial apoptosis pathway is induced by hsp90 translocation into the mitochondria through interacting with hypoxia-responsive pro-apoptotic protein (Arya et al. 2007). Hsp110 can be a caspase activator as it stimulates the apoptosome (Cande et al. 2002).

Various stressors can result in an increase in extracellular heat shock proteins. Physical trauma, behavioural stress, and reaction to immune signals can result in release of hsp27, hsp60, hsp70, grp78, hsp90 and hsp110 from cells. These extracellular hsps can interact with adjacent cells or in the case of hsp60 and hsp70 enter the bloodstream. They function to initiate signal

transduction cascades and transport antigenic peptides. Extracellular hsps have been found to have immune effects. Extracellular hsp60 has been found to be pro-inflammatory while extracellular hsp27 and grp78 have been found to be anti-inflammatory (Calderwood et al. 2007).

Various hsp are involved in the actions of the immune system. Grp78 is involved in the initiation of folding of major histocompatibility complex (MHC) I and II (Zugel and Kaufmann 1999). Extracellular grp78 and hsp27 have been found to have anti-inflammatory properties (Calderwood et al. 2007). Hsp70 is involved in the processing and presentation of antigens (Zugel and Kaufmann 1999). Hsp70 has been found to increase inducible NO synthase (iNOS) and increase NO release from activated antigen presenting cells (APC) (Panjwani et al. 2002). Extracellular hsp60, conversely, has been found to be pro-inflammatory and may be involved in shifting immune response toward T helper (Th)1 responses via its induction of interferon (IFN)-γ and IL-12 following stress exposure (Breloer et al. 2001, Calderwood et al. 2007). It has been hypothesized that extracellular hsp70 may be involved in limiting the reaction to self-antigens following tissue damage (Panjwani et al. 2002). Hsp60 and hsp70 have also been implicated in the development of autoimmunity (Zugel and Kaufmann 1999).

Other than the hsp families described above, another important cellular stress protein is cytokeratin. Depending on the cellular context, cytokeratin can impact cellular size, architecture, proliferation and tissue integrity. Cytokeratin protects cells from mechanical stress and plays a role in cellular migration and differentiation (Magin et al. 2007). Through cell to cell contacts cytokeratin play a role in epithelia integrity and mechanical stability (Moll et al. 2008). The keratin cytoskeleton is made up of type I and type II cytokeratins which are part of the cell's intermediate filaments (Magin et al. 2007). Within the epithelium the cytokeratins present depend on the cell type and the stage of cellular differentiation. Hair follicles and fibers within the skin also have many cytokeratins (keratin (K) 25-28 and K71-75, and K31-40 and K81-86, respectively) (Moll et al. 2008). Certain cytokeratins are inducible (K6, K16 and K17) while some are up and down regulated following tissue wounding (K1-10) (Magin et al. 2007). Cytokeratin intermediate filaments are involved in the resistance to various stresses and apoptosis, such as osmotic and hypo-osmotic stress and starvation. It has been suggested that cytokeratins sequester stress-activated phosphate kinases thereby protecting the cell from injury.

Cytokeratins can interact to moderate death receptor and cell-intrinsic apoptosis pathways (Magin et al. 2007). Cytokeratins may be affected by glucocorticoids as several isoforms have negative glucocorticoid response elements within their DNA sequence (Dostert and Heinzel 2004).

### 1.7.4 Oxidative stress and inflammation proteins

Stress can affect immune function. The magnitude of the effect depends on the type of stress, intensity and duration (Spencer et al. 2001). Glucocorticoids are thought to produce a shift in immunity with downregulation of Th1 cytokines and upregulation of Th2 cytokines (Webster Marketon and Glaser 2008). Glucocorticoids suppress the production of pro-inflammatory cytokines and up-regulate various anti-inflammatory cytokines. Glucocorticoids suppress maturation, differentiation and proliferation of innate immune cells (monocytes), T cells and B cells (Webster et al. 2002).

The duration of stress exposure affects whether shifting immunity is harmful or beneficial for an organism. Acute stress generally brings about immunoenhancement, while chronic stress is associated with immunosuppression (Dhabhar 2000). The advantages of the selective suppression of the immune system by chronic stress include a check on inflammation which can be damaging to tissues and suppression of the lethargy and other sickness behaviours that allow animals to heal, but might interfere with coping with or escaping the current chronic stressor(s) faced. Immunosuppression associated with chronic stress is not absolute however; as there is evidence that in some cases of repeatedly applied and chronic stress there is an attenuation of glucocorticoid immunosuppression resulting in immunoenhancement (Black 2002, Nakano 2004). Chronic stress has been found to be associated with a rise in pro-inflammatory cytokines (Leonard 2005). In humans, chronically elevated glucocorticoids are associated with protracted wound healing and increased susceptibility to viral infections (Webster et al. 2002). In cattle, stressors such as transportation, weaning, restraint, cold and social organization were found to increase susceptibility to infection with viruses and bacteria (Aich et al. 2007). Increased disease severity in captive compared to free-ranging cheetahs (*Acinonyx jubatus*) was hypothesized to result from chronic stress (Munson et al. 2005).

Oxidative stress is the imbalance between production of free radicals and reactive metabolites and antioxidant systems that can lead to damage of biomolecules and organs (Durackova 2010). Reactive oxygen species include radicals and metabolites, such as nitric oxide, superoxide and hydroxyl radical (Durackova 2010). Nitric oxide synthases produce NO within cells and are important for tissue integrity. Maintenance of skin barrier function and blood flow rate to the microvasculature seem to depend on low level, constitutive NO production. Higher levels of NO production, generated by endothelial NO synthase (eNOS) and inducible NO synthase (iNOS), are involved in wound healing (Cals-Grierson and Ormerod 2004). At one cellular concentration NO can be protective to the cell and at a higher concentration it can promote apoptosis (Cals-Grierson and Ormerod 2004). Most of the vascular NO is produced by eNOS (Forstermann 2006). The epidermis and dermis are comprised of a variety of cells that have been found to express iNOS. Melanocytes, fibroblasts, microvasculature, arrector pili muscle, eccrine coiled duct, apocrine and eccrine secretory glands and hair follicles have been found to express eNOS (Cals-Grierson and Ormerod 2004). Cytokines or LPS can induce iNOS (IFN-γ, IL-1β, TNF-α) and after induction it produces NO until it is degraded (Guzik et al. 2003, Kleinert et al. 2004). Both Th1 and Th2 cells produce NO and their expression may be inhibited by it (Guzik et al. 2003). Elevated levels of NO can be pro-inflammatory (Cals-Grierson and Ormerod 2004). Antiviral, antimicrobial, and antiparasital effects have been attributed to NO (Kleinert et al. 2004). Nitric oxide and iNOS are influenced by and can influence the stress axis. Glucocorticoids, tumor growth factor-β, IL-4 and IL-10 can inhibit the induction of iNOS (Guzik et al. 2003). Nitric oxide can inhibit CRH-induced ACTH secretion and corticosterone secretion (Guzik et al. 2003). Nitric oxide production can have negative consequences within cells. Nitric oxide reacts with superoxide to form peroxynitrite which can interact with proteins, lipids, carbohydrates and DNA leading to oxidative damage of tissues (Aktan 2004).

The immune response and oxidative stress of tissues are influenced by different forms of heme oxygenase (HO). Products of HO activity include biliverdin and Fe. Biliverdin inhibits viral replication and bilirubin, the product of biliverdin, is a potent antioxidant (Maines 1997). Both biliverdin and bilirubin inhibit inflammatory responses. Heme oxygenase 2 (HO2) has been suggested as a possible “sink” for NO and gaseous heme ligands (Maines and Panahian 2001). Heme oxygenase 2 (HO2) levels are increased by glucocorticoids (Maines 1997).

However, long-term restraint stress results in decreased HO2 concentration in the hippocampus (Chen et al. 2005).

Superoxide dismutase (SOD) and peroxiredoxin (PRDX) are part of the evolutionarily conserved cellular response to oxidative stress (Kultz 2005). Superoxide anions are converted by superoxide dismutase to hydrogen peroxide, which is then converted to water by peroxiredoxin (Kultz 2005). The isoform SOD1 (Cu/Zn SOD) is found in cytoplasm, while SOD2 (Mn SOD) is found in the mitochondria (Zelko et al. 2002). Both SOD1 and SOD2 are constitutively expressed and mRNA levels have been found to dramatically fluctuate under various physiological conditions. Cellular exposure to heavy metals, NO, hydrogen peroxide and hypoxia has been found to cause fluctuations in SOD1 levels. Exposure to cytokines, such as IL-1, IL-4, IL-6 and TNF-α has been associated with fluctuations in SOD2 (Zelko et al. 2002). There is evidence to suggest that SOD2 protects mitochondria functionality from oxidative stress (Kokoszka et al. 2001). Reactive oxygen species are produced to defend against invading bacteria during skin wound healing. Both SOD1 and SOD2 have been found to be involved in detoxification of ROS at wound sites, with SOD1 upregulation lasting longer than SOD2, up to 7 days (auf dem Keller et al. 2006). Peroxiredoxins are part of the cellular adaptation to oxidative stress (Kultz 2005). There are six mammalian PRDX isoforms (Rhee et al. 2005). Peroxiredoxin 3 is a mitochondrial scavenging enzyme for hydrogen peroxide. Hydrogen peroxide, although a weak oxidant, is readily converted to the more powerful oxidant, hydroxyl radical, by the Fenton reaction. Depletion of PRDX3 was found to result in increased hydrogen peroxide concentrations and an acceleration of apoptosis triggered by TNFα (Chang et al. 2004). Maintaining normal mitochondrial function may require PRDX3 (Wonsey et al. 2002).

CC chemokine receptor 5 (CCR5) is involved in immune functioning. Regulating trafficking and effector functions, CCR5 is found in memory/effector Th1 cells, immature dendritic cells, macrophages and natural killer cells, and is involved in viral pathogenesis and inflammatory diseases. Migration of monocytes, natural killer cells and Th1 cells towards inflammation sites involves CCR5 (Balistreri et al. 2007). CC chemokine receptor 5 is suspected to be one of the cell surface receptors involved in mediating the effects of extracellular hsp70 (Calderwood et al. 2007).

Cyclooxygenase (COX) enzymes are involved in prostaglandin production, which affects a wide variety of physiological processes. The first two steps of prostanoid biosynthesis are catalyzed by the COX isozymes COX1 and COX2 (Zaric and Ruegg 2005). A range of normal physiological processes involve prostaglandins, such as vasomotor tone, platelet aggregation, differentiation of immune cells, nerve growth, wound healing, bone metabolism, renal function, initiation of labour, ovulation and kidney function (Dubois et al. 1998, Steer and Corbett 2003). COX2 is thought to have a pro-inflammatory role in an acute immune reaction which switches to an anti-inflammatory role as the reaction resolves (Willoughby et al. 2000). Prostaglandins participate in regulation of viral replication and modulation of inflammatory response (Steer and Corbett 2003). Viral infection, inflammation and the release of inflammatory cytokines, such as TNF, IL1β and IL6, results in an increase in COX2 expression (Dubois et al. 1998, Steer and Corbett 2003, Zaric and Ruegg 2005). Anti-inflammatory cytokines, such as IL4 and IL9, have been found to inhibit COX2 expression (Hinz and Brune 2002). Expression of iNOS and COX2 are thought to be coordinately regulated under certain inflammatory conditions (Steer and Corbett 2003). Stress affects COX2 expression, since glucocorticoids have been shown to inhibit the production of COX2 (Sapolsky et al. 2000).

### 1.8 Stress protein expression as an "early warning" of animal health

In developing a protein microarray to measure expression patterns of multiple stress-associated proteins in wildlife such as grizzly bears, the goal is not to develop a replacement for traditional field techniques, as they provide vital information, but instead to develop a unique tool to rapidly determine which populations may need the most attention, so as to better allocate limited resources and provide an early warning that a wildlife population is at risk. This technique may thus provide more information about stressed populations, which will be useful for management decisions. The rapid assessment of individual grizzly bear health using a stress protein microarray may provide justification for use of other, more labour intensive approaches. Additionally, in certain endangered and/or protected species this may provide the only glimpse at the health of the population.

Some wildlife techniques may carry risk to specific species. For example, female brushtail possums (*Trichosurus vulpecula*) handled frequently by researchers were found to have lower survival as did their young prior to the age of weaning (Clincy et al. 2001). Long

immobilization during capture has been linked to reduced female fertility of black rhinoceros (*Diceros bicornis*) (Alibhai et al. 2001). There is also concern about chemical immobilization reducing production of young and increasing abandonment of young in mountain goats (*Oreamnos americanus*) (Cote et al. 1998), although in other species no effect of immobilization was observed (Cote et al. 1998, Creel et al. 1997). Age-specific body condition was found to be generally poorer in grizzly bears captured more than once (Cattet et al. 2008a). Previously captured grizzly bears were reported have decreased movement rates for up to 3-6 weeks and a small proportion of bears were found to have evidence of muscle injury in the limb snared by the leg-hold snare (Cattet et al. 2008a). A tool that would allow evaluation of chronic stress in wildlife without the need of capture or immobilization would be a valuable addition to a wildlife researcher's arsenal. If a microarray indicated increased stress, it may be a strong indicator that more scientific study using other research tools is needed.

A more comprehensive picture of the problems encountered by grizzly bears is required for grizzly bear conservation. Without adequate information of the difficulties faced by these animals, wildlife managers and other concerned individuals will not be able to effectively allocate or mobilize limited resources (Mattson et al. 1996). There has been a call in the literature for the development of new techniques to assess bear population health, because of the uncertainties that exist with current methods (Garshelis 2002). The increase in ecological understanding of wildlife health issues is needed in order to facilitate forecasting of the effects of ecological change and assist decision-making process (Clark et al. 2001). The proteomic technique developed in this thesis will add valuable information regarding the health status of bear populations and in the future may be adapted to use for other wildlife species, thus making its application and significance more extensive.

### 1.9 Hypothesis and goal of thesis

The stress response can be beneficial for reasons varying from avoiding physical attack to fending off infection or parasite invasion. The danger exists when an animal is subjected to long-term stress. Some stress systems cause direct tissue damage when in excess. Chronic stress can lead to inhibition of growth, immune function and reproduction that is not only detrimental to individual bear health, but also has the potential to have adverse effects at the population level. Determining whether long-term stress is influencing a grizzly bear population with lower than

normal reproductive rate would be a vital contribution towards forecasting the continued health of these populations. The **general working hypothesis** of my thesis is that long-term stress caused predominantly by landscape change can negatively affect individual grizzly bear health, thereby negatively affecting the sustainability of bear populations.

The **overall objective** of my research was to develop a protein microarray that detects long-term stress in tissue (skin or muscle) biopsy samples collected from free-ranging grizzly bears. The goal was to develop a technique to measure a suite of stress-associated proteins that may be up- or down-regulated in individual bears, thus providing a monitoring tool to assist wildlife managers in evaluating bear populations at potential risk. This tool was used to determine if the patterns of stress protein expression could be correlated with decreased condition in bears or human activity within the bear's home range, such as road density, anthropogenic change and the proportion of habitat protected, thus creating a new tool for detecting problems in wildlife population health. Ideally, the stress protein expression patterns will help elucidate whether the grizzly bears in the foothills of Alberta are experiencing long-term stress, and what specific changes in bear health are occurring. This information may help in elucidating why certain grizzly bears in Alberta have a lower reproductive rate than grizzly bears in other areas (Garshelis et al. 2005).

## Chapter 2 Methods

### 2.1 Animal capture and sample collection

The Foothills Research Institute (FRI) Grizzly Bear project captured grizzly bears in western Alberta encompassing the area from the Montana border north into the boreal forest and centrally to the Swan Hills from 2004 to 2008. Captures mainly occurred between May and June, but trapping began in March and ended in November. For my thesis research, n=133 individual bears were captured. Bears captured by culvert trap included 16 females, 24 males and 2 bears whose sex was not determined. Bears captured by helicopter dart included 12 females and 12 males. Bears captured by snare included 22 females and 45 males. Sex was determined by examination of external genitalia. Capture methods included leg-hold snare, remote drug delivery from helicopter and culvert traps (see Cattet 2008 for a detailed capture and handling protocol). Accessibility and terrain openness were used to choose which capture method was used (Hobson 2006, Hobson et al. 2008). Skin and muscle samples were collected from captured bears using biopsy needle, biopsy punch or biopsy dart and frozen as soon as possible at -20$^{o}$C. This protocol was approved by the University of Saskatchewan University Committee on Animal Care and Supply each year for the duration of the project. The protocol was in accordance with guidelines of the American Society of Mammalogists Animal Care (1998) and Use Committee and the Canadian Council on Animal Care (2003).

In 2004, muscle biopsies were taken using a Tru-cut biopsy needle (Travenol Labouratories Inc., Morton Grove, IL, USA) from the quadriceps muscles of anesthetized bears by a veterinarian in the field. From 2005-2008, one to six 4mm diameter skin samples were collected by sterile disposable biopsy punch (Miltex Inc., York, PA, USA) from different areas of the body (neck, left forelimb, right forelimb, left hindlimb and right hindlimb). Before sampling, either skin or muscle, a small area (2-3cm diameter) was shaved with rechargeable clippers. The biopsy site was scrubbed with Povidone-iodine and alcohol and then 3-5mL of 2% Lidocaine with epinephrine was injected at the biopsy site. Muscle samples were taken by making a 2.5mm incision using a scalpel to permit the use of the Tru-cut biopsy needle. If necessary the wound was closed using one or two interrupted sutures (3-0 coated Vicryl). Skin samples were also collected by biopsy dart (Paxarms NZ Ltd., Timaru, NZ) from the left hindlimb in bears captured by leg-hold snare. A Paxarms biopsy dart (5mm long x 4 mm wide

cutting head) was fired at close range into the thigh of an anesthetized bear. The darts fell from the skin post impact.

In addition to the small (approximately 50-100 mg) skin and muscle samples collected from captured bears, larger pieces of skin and muscle (approximately 1-2 kg) were opportunistically collected from six bears that died through bear management measures, self defense actions or were killed by another predator. In these latter samples, each bear carcass was at ambient temperature for between 2 to 12 hours before storage of tissue was possible. The larger bulk quantities of tissue from these bears were used for development of the stress protein microarray, as described below.

In order to determine if there was a difference in skin protein expression depending on the location of samples, thirty-one bears had multiple skin samples from varying locations taken at time of capture. From three bears muscle and skin samples were collected for comparative analysis. From 2004-2008, each bear was fitted with an ear-tag radio transmitter. After shaving a small ($4cm^2$) area, a 6mm biopsy punch (Miltex, Inc., York, PA, USA) was then used to make a hole in the ear to allow the ear-tag to be applied. The ear punch biopsies were taken and frozen for stress protein microarray analysis.

**2.2. Sample preparation**

Microarray development was approached from three angles, using the bulk skin and muscle samples described above: (1) testing of commercially available antibodies for their cross-reactivity with specific grizzly bear proteins, and thus utility in the microarray, (2) two dimensional polyacrylamide gel electrophoresis (2D-PAGE) of grizzly bear skin and muscle samples followed by mass spectrometry of individual proteins, in order to identify proteins that were significantly up- or down-regulated following stress, and (3) evaluation of three commercially available antibody-based protein microarrays developed for human biomedical (clinical diagnostic) applications. Once a suitable panel of stress-associated proteins were selected using these approaches, the antibodies were used to create a custom protein microarray.

For the western blotting and microarrays, skin and muscle samples were processed using a modification of established methods (Haab and Zhou 2004). For the 2D-PAGE, skin and

muscle samples were processed using a modification of established methods (Choudhury et al. 2006 and Doran et al. 2004 respectively).

### 2.2.1 Sample processing for microarray and western blotting

Grizzly bear samples, stored at -80˚C, were homogenized to powder by freezing in liquid nitrogen and ground using mortar and pestle. In the case of skin samples, all hair was removed prior to homogenization. Proteins were isolated from the homogenized samples by adding 10 ml lysis buffer (50 mM HEPES buffer pH 7, 5 mM EDTA, 50 mM NaCl, 10 mM sodium pyrophosphate, 50 mM NaF, 10 mM sodium vanadate, 1% Nonidet P40, complete protease inhibitor (Roche, Toronto, ON, Canada) per gram of tissue and incubating 15 minutes on ice. After centrifuging the lysed samples, the supernatant was collected and concentrated using dialysis membrane (Spectra/Por Biotech regenerated cellulose dialysis membrane 133-110, Spectrum Labouratories, Inc., Rancho Dominguez, CA, USA) or centrifugal filters (Ultracel YM-10, catalog no. 42408, Millipore Corp., Bedford, MA, USA) and stored at -80˚C.

### 2.2.2 Sample preparation for two dimensional polyacrylamide gel electrophoresis

Frozen grizzly bear skin samples were homogenized to powder by freezing in liquid nitrogen, removing all hair to skin level and ground using mortar and pestle. Proteins were isolated from the homogenized samples by adding 5ml per gram tissue of lysis solution (10 mM Tris pH 8.0, 5 mM magnesium acetate, 8 M urea, 2M thiourea, 4% CHAPS and Complete wide-spectrum protease inhibitor Mini (Roche)). Samples were incubated for 1 hr then briefly sonicated at room temperature. Samples were then centrifuged at 4$^{o}$C for 15 minutes at 7500g. The supernatant was collected and diluted with rehydration solution (2M thiourea, 8M urea, trace coomassie brilliant blue, 0.1% Tergitol NP7, 2% CHAPS, Complete wide-spectrum protease inhibitor Mini). This solution was incubated for 30 minutes and then centrifuged at 11,300g for 2 minutes at 4$^{o}$C. The supernatant was then collected and frozen at -80˚C for subsequent 2-D gel electrophoresis.

Frozen grizzly bear muscle samples were homogenized to powder by freezing in liquid nitrogen and ground using mortar and pestle. Proteins were isolated from the homogenized samples by adding 5ml per gram tissue of cold buffer A (0.175 M Tris HCl pH 8.8, 5% (w/v) SDS, 15% (v/v) glycerol, 0.3 M dithiothreitol, protease inhibitor cocktail including 1 mM EDTA

(Roche), 2 µl DNase I / 100 µl buffer). The homogenate was then filtered through 2 layers of miracloth (Calbiochem 475855, EMD Bioscience, Gibbstown, NJ, USA). Four volumes ice-cold 100% (v/v) acetone was added and the mixture was mixed by vortexing. The sample was incubated for 1 hour at -20ºC to precipitate the protein. The sample was centrifuged at 5000g for 15 minutes at 4°C. The pellet was washed with 20 ml ice-cold 80% (v/v) acetone. The sample was vortexed, sonicated and washed again. The sample was centrifuged and resuspended by gently vortexing and adding 1 ml buffer B (10 mM Tris pH 8.0, 5 mM magnesium acetate, 9.5 M urea, 4% CHAPS). The sample was incubated for 3 hours at room temperature, vortexing every 10 minutes for 5 seconds. The samples were then centrifuged for 20 minutes at 20,000g and 4°C. The supernatant was collected as the sample to be used for 2D-PAGE.

#### 2.2.2.1 Keratin stripping of skin samples for 2D-PAGE

Keratin stripping was performed on the skin samples prior to running on 2D-PAGE in 2006. Removal of abundant proteins enables one to detect less abundant proteins with 2D-PAGE (Girault et al. 1989, Shaw and Riederer 2003). Briefly, the cytokeratin antibody (Abcam ab9377, Cambridge, MA, USA) buffer was exchanged by concentrating the antibody (Millipore micron ultracel YM-3 cat#42420), centrifuged at 13,000g at 4°C for 60minutes, and then resuspended in 7mM HEPES coupling buffer. Affigel 10 (Bio-Rad 153-6099, Hercules, CA, USA) was placed in a column rinsed with 3 volumes of 10mM cold sodium acetate and then incubated in a 5mL tube with antibody solution. The tube was rotated on platform for 4 hours at 4°C. The gel mix was then transferred back to the column and washed with coupling buffer 6 times. The last wash was collected and protein content was determined to ensure no protein had eluted. The sample was then added to the funnel, and unbound sample was eluted with 0.5% Triton X-100 and collected. The protein concentration was then determined (RCDC kit, Bio-Rad) and the samples were diluted to 1µg protein/µl with buffer B (13mM Tris base pH 8.0, 18mM magnesium acetate, 9M urea, 65mM CHAPS). Unless otherwise stated, protein concentrations were determined using DC Bio-Rad Protein Assay (Bio-Rad), which is a modification of Lowry et al. (1951).

## 2.3 Two dimensional polyacrylamide gel electrophoresis

Two dimensional gel electrophoresis was used as a tool to identify proteins altered in bears paired by age and sex, but thought to be experiencing different levels of stress. In 2006 and 2007 2D-PAGE was completed following a protocol in the 2-D electrophoresis handbook of GE Healthcare (Piscataway, NJ, USA). The 2D-PAGE procedure was performed at the Institute of Biomolecular Design (IBD, Edmonton, AB, Canada) in 2006 and at the University of Waterloo (Waterloo, ON, Canada) in 2007. Proteins of interest from 2006 and 2007 underwent mass spectrometry and identification using MALDI-Tof/Tof and Q-Tof mass spectrometry and Mascot software at the IBD.

The two dimensional electrophoresis procedures for 2006 and 2007 were conducted according to the protocol of GE Healthcare (Piscataway, NJ, USA). Determination of differentially expressed proteins and sample extraction methods were different for each year. In 2006, two muscle samples and four skin samples were taken to the Institute of Biomolecular Design. Pairs of samples from bears with putatively less and greater stress were compiled using the health and landscape data collected when the bears were sampled. Three 2-dimensional electrophoresis experiments were run, one for the muscle pairing and two for the skin samples. Each sample was dyed with fluorescent dyes, so that each gel had one sample dyed with cyanine (Cy) 3 and one sample dyed with Cy5. A pool of the skin samples dyed with mono-reactive NHS ester cyanine fluorophores Cy2 (GE Healthcare) was used as an internal reference for the gels that contained skin samples and a similar pool of muscle was labeled with Cy2. Protein expression pattern differences were evaluated by comparing the fluorescence of Cy3 and Cy5 for each protein in the gel, scanned with a GE Typhoon Imager. For the 2006 samples elevated or depressed expression was determined as 5 to 10 fold difference in expression. For the 2007 samples elevation and depression of expression was determined by the DeCyder software. The spot picking was done on the same gels stained with Coomassie with an automated Perkin Elmer MassPrep Station (Waltham, MA, USA). Proteins of interest were tryptic digested in gel. Briefly, proteins in the gel were de-stained, reduced (DTT), alkylated (iodoacetamide), and digested with trypsin (Promega Sequencing Grade Modified). Digested proteins underwent mass spectrometry using MALDI-Tof/Tof and Q-Tof mass spectrometry Protein identities were determined using Mascot software (Matrix Science Inc., Boston, MA, USA).

Two-dimensional polyacrylamide gel electrophoresis was also conducted in 2007 at the University of Waterloo. The method was the same as above until the analysis of the 2nd dimension gel. The scanning was again conducted with a Typhoon imager but this time DeCyder software (GE Healthcare) was used, which was able to determine significant differences between paired samples (t-test, $p<0.05$). Additional duplicated gels were then run with unlabeled samples. The gels were Coomassie stained and the spots with significant changes were picked manually. The gel pieces were frozen and shipped to the Institute for Biomolecular Design where they went through the same in gel digestion and identification as the 2006 samples.

### 2.4 Commercial microarrays

Three commercial antibody-based protein microarrays were tested using skin samples processed following the method of Haab and Zhou (2004). The same skin samples from the 2D-PAGE work described above were used. The arrays tested were Hypromatrix Signal Transduction antibody array (Hypromatrix, Worcester, MA, USA), Spring Bioscience antibody microarray (Spring Bioscience, Fremont, CA, USA) and Sigma Panorama Antibody Cell Signaling array (Sigma Aldrich, St. Louis, MO, USA). The same tissue processing technique described above was used except blocking agents and wash buffers provided by suppliers were used. Slides were scanned with an Affymetrix Array Scanner (Affymetrix, Santa Clara, CA, USA) with Jaguar software. The resulting images were processed by ArrayVision software, and fold differences were determined by ArrayPipe software (Genome Canada Pathogenomics Project, Vancouver, BC, Canada). The antibodies that showed two-fold or greater differences in binding when comparing one sample to another were then validated for use in the customized grizzly bear microarray using western blotting, as described below. The samples run for the western blotting were processed using the same technique as the microarray samples.

### 2.5 Antibody testing

As all of the antibodies tested were raised against proteins from non-bear species (human, mouse, rat, etc.), a large number of antibodies (285) from 19 companies were screened using western blotting (Towbin et al. 1979) to obtain 32 antibodies for subsequent microarray development with strong signal and low background (i.e., non-specific binding). Isolated

proteins from bulk bear tissue samples were denatured and separated by size with SDS-PAGE using 7.5%, 12.5% or 15% acrylamide gels. Proteins larger than 100kDa were separated with 7.5% gels, while those smaller than 40kDa were separated with 15% gels. Proteins in the gel were then transferred to a 0.22 μm nitrocellulose membrane (PVDF Transfer membrane Hybond-P, GE Healthcare) and hybridized with a dilution of a chosen antibody. Hybridization of the antibody was detected using enhanced chemiluminescence (ECL Plus Western Blotting detection reagents RPN2133, GE Healthcare) wherein a secondary horseradish peroxidase-conjugated antibody (rabbit anti-goat IgG ab6741 [Abcam, Cambridge, MA, USA], donkey anti-mouse 6410-05 [Southern Biotech, Birmingham, AL, USA], or donkey anti-rabbit 6440-05 [Southern Biotech]) was hybridized to the bound primary antibody and the enzymatic chemiluminescence reaction was subsequently initiated and detected using autoradiography film. Based upon initial results, the primary antibody dilution was optimized and each antibody re-tested. The size of the resultant band(s) was determined through comparison to molecular standards (Kaleidoscope prestained standards 161-0324 or Precision Plus Kaleidoscope 161-0375, Bio-Rad**)** and only those antibodies that recognized protein bands of the correct molecular weight were selected as positive. Additionally, those antibodies that recognized additional nonspecific bands were rejected.

### 2.6 Antibody printing and microarray production

Microarrays were printed (i.e., antibodies immobilized onto glass slides) by First Phase Technologies (Tempe, AZ, USA) onto Full Moon BioSystems (Sunnyvale, CA, USA) protein array substrate (PTR) slides. Before the final slides used to determine stress protein expression in individual bears were printed, a set of dilution slides were printed. These slides consisted of a dilution series of antibodies diluted 1:625, 1:125, 1:25 and 1:5 with printing buffer and one undiluted spot. The antibodies printed were hsp60 antibody (h3524, Sigma-Aldrich), caspase 2 antibody (RB-1699, Lab Vision, Fremont, CA, USA), ACTH antibody (E54057M, Biodesign, Saco, ME, USA), cytokeratin antibody (ab9377, Abcam), caspase 3 antibody (sc-1225, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), POMC antibody (ab32893, Abcam), and hsp70i antibody (SPA-810, Stressgen Biotechnologies Corp., Victoria, BC, Canada). Also printed on each array were a dye labeled protein selected by First Phase Technologies diluted 1:625, 1:125, 1:25, 1:5 and 1:1 with printing buffer and four spots of print buffer. These

preliminary microarrays were used to test methods of isolating replicate arrays on each slide, blocking buffers, wash buffers, and time of incubation.

Once the initial validation was completed, final protein microarrays were produced. Six replicate microarrays were printed onto each slide, with each array consisting of 36 spots in a 6x6 grid consisting of 31 distinct antibodies specific for grizzly bear stress proteins, antibody that did not recognize bear proteins (a spike control), a print buffer-only spot (negative control), and a dye-labeled protein spot (positive control, provided by First Phase Technologies) (Table 2.1). Among the antibodies printed onto the array was a dilution series of an anti-cytokeratin antibody printed at dilutions of 1:1, 1:5, and 1:25 in print buffer. Other antibodies were diluted 1:1 in print buffer. Once printed, arrays were stored at room temperature in a sealed desiccator until use. Six separate print runs were used to generate all the slides necessary. Slides were placed in a randomized sequence to minimize the contributions of print run variation.

**Table 2.1** Antibodies chosen for the grizzly bear microarray chip

| Antibody | Monoclonal/ polyclonal | Supplier | Catalog # | Host Animal | Antigen Source |
|---|---|---|---|---|---|
| Adrenocorticotropic hormone (ACTH) | M | Biodesign | BDE54057M | Mouse | Human |
| Apoptosis Inducing Factor (AIF) | M | Santa Cruz | sc-13116 | Mouse | Human |
| Annexin II | P | Santa Cruz | sc-1924 | Goat | Human |
| Annexin IV | P | Santa Cruz | sc-1930 | Goat | Rat |
| Arginine Vasopressin (AVP) Receptor V1a | P | Santa Cruz | sc-30025 | Rabbit | Human |
| Caspase 1 | P | Santa Cruz | sc-514 | Rabbit | Mouse |
| Caspase 2 | P | Lab Vision | RB-1699 | Rabbit | Human |
| Caspase 3 | P | Santa Cruz | sc-1225 | Goat | Human |
| Caspase 6 | | Sigma | C7599 | Rabbit | Human |
| Chemokine (C-C motif) receptor (CCR5) | | Sigma | C8604 | Rabbit | human |
| Cyclooxygenase (Cox) 2 | P | Santa Cruz | sc-7951 | Rabbit | Human |
| Corticotropin-Releasing Hormone Receptor (CRHR) 1/2 | P | Santa Cruz | sc-5543 | Rabbit | Human |
| Cytokeratin | P | Abcam | ab9377 | Rabbit | Bovine |
| Epithelial (E) Cadherin | P | Santa Cruz | sc-31020 | Goat | Human |
| Endothelial Nitric Oxide Synthase (eNOS) | P | Abcam | ab5589 | Rabbit | Human |
| Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) | P | Assay Designs | 905-734-100 | Rabbit | human |
| Glucocorticoid Receptor (GR) | P | Santa Cruz | sc-1002 | Rabbit | human |
| Glucose Regulated Protein (grp) 78/BiP | | Sigma | G9043 | Rabbit | human |
| Heme Oxygenase (HO) 2 | P | Santa Cruz | sc-11361 | Rabbit | Human |
| Heat Shock Protein (hsp) 110 | | Sigma | H7412 | Rabbit | human |
| Heat Shock Protein (hsp) 27 | P | Stressgen | SPA-524 | Rabbit | Human |
| Heat Shock Protein (hsp) 40 | | Sigma | H4038 | Rabbit | human |
| Heat Shock Protein (hsp) 60 | M | Sigma | H3524 | Mouse | Human |
| Heat Shock Protein (hsp) 70 | M | Santa Cruz | sc-24 | Mouse | Human |
| Inducible Heat Shock Protein 70 (hsp70i) | M | Stressgen | SPA-810 | Mouse | Human |
| Heat Shock Protein (hsp) 90 | P | Stressgen | SPS-771 | Rabbit | Mouse |
| Inducible Nitric Oxide Synthase (iNOS) | | Sigma | N7782 | Rabbit | mouse |
| c-terminal Proopiomelanocortin precursor (POMC) | P | Abcam | ab32893 | Goat | Human |
| Peroxiredoxin (PRDX3) | | Sigma | P1247 | Rabbit | human |
| Prolactin | P | Santa Cruz | sc-7805 | Goat | Human |
| Superoxide Dismutase (SOD) 1 (Cu/Zn) | P | Santa Cruz | sc-8637 | Goat | Human |
| Superoxide Dismutase (SOD) 2 (Mn) | P | Abcam | ab13533 | Rabbit | human |

## 2.7 Antibodies categorized by functional category

Of the 37 antibodies validated by western blotting to be specific for grizzly bear skin and/or muscle, 31 were selected for the grizzly specific array. The proteins were divided into 4 groups: HPA axis, apoptosis and cell cycle, cellular stress, and oxidative stress and inflammation based on their biological function and relationship to stress (Table 2.2). Caspase 3 was chosen as a control antibody.

**Table 2.2** Physiological function categories of the protein microarray antibodies on the custom grizzly bear microarray. Each antibody is listed with its full protein target name, abbreviated name.

| **Category** | **Protein** |
|---|---|
| Hypothalamic-pituitary-adrenal axis | adrenocorticotropic hormone (ACTH), arginine vasopressin receptor (AVP) Receptor V1a, corticotropin-releasing hormone receptor (CRH)-Receptor 1/2 (CRHR-1/2), glucocorticoid receptor (GR), C-terminal proopiomelanocortin (POMC) precursor, prolactin |
| Apoptosis and cell cycle | apoptosis inducing factor (AIF), annexin II, annexin IV, caspase 1, caspase 2, caspase 6, epithelial (E)-cadherin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) |
| Cellular stress | cytokeratin, glucose regulated protein (grp78 / BIP), heat shock protein (hsp)110, hsp27, hsp40, hsp60, hsp70, hsp70 inducible (i), hsp90 |
| Oxidative stress and inflammation | chemokine (CC-motif) receptor (CCR) 5, cyclooxygenase (COX)2, heme oxygenase (HO)-2, endothelial nitric oxide synthase (eNOS), inducible (iNOS), peroxiredoxin 3 (PRDX3), superoxide dismutase (SOD)1, SOD2 |

## 2.8 Pooled standard

To normalize slide to slide variation, a pooled standard was run on each slide. The fluorescence of each individual bear sample was divided by (compared to) the fluorescence of the pooled standard. One large pool of sample was made up from skin from the six large samples available. Each bear's skin was homogenized separately. At the end of the tissue processing an equal amount of protein from each of the six bears was added to make the pooled standard. The pooled standard was then separated into 300µl aliquots and frozen.

## 2.9 Protein labeling

In order to detect the proteins that were specifically bound to the antibodies on the array, proteins were labeled with fluorescent dyes. Proteins for microarray hybridization were labeled using the synthetic cyanine dyes, Cy3 and Cy5, following the protocol provided with the dyes (GE Healthcare). Individual bear samples were labeled with Cy5, and the pooled standard labeled with Cy3.

For the labeling reaction, Cy dyes were dissolved in DMSO to 1.5 mM. Samples were labeled by incubating on ice with 300 µM of each Cy dye in 50mM carbonate buffer (pH8.5), and the reaction was quenched after incubation with the addition of 1M Tris-HCl (1/10 total reaction volume). Excess dye was subsequently removed from the labeled protein using 10DG disposable chromatography columns (Bio-Rad Labouratories cat. no. 732-2010 or Microcon Ultracel YM-10, Millipore cat. no. 42408). Labeled protein samples were concentrated using centrifugal filters, and protein assays (Lowry et al. 1951) were conducted to determine the protein concentration of the labeled individual bear sample and the labeled pooled standard.

## 2.10 Microarray hybridization

Silicone isolators were clamped onto microarray slides (Grace Bio Labs, cat. no. 204862, Bend, OR, USA) to separate the six replicate arrays and create discrete wells on the slide. Arrays were blocked prior to use by incubation with 1% bovine serum albumin (BSA, 2910-OP EMD Biosciences, Gibbstown, NJ, USA) for 30 minutes, rinsed with $ddH_2O$, then washed 5 times for 5 minutes with phosphate buffered saline with 0.5% Tween-20 (0.5% PBST, pH 7.4), 3 more times for 5 minutes with $ddH_2O$ and finally dried under a stream of $N_2$. Equal amounts of

protein (80 µg) from the labeled pooled standard and test sample were combined, diluted with 5% BSA, and then added to each of three arrays on a slide, with two samples applied per slide. Thus each sample was run in triplicate if sufficient sample protein was available. The hybridization reaction was incubated for 1 hour with agitation before the samples were removed from the arrays and rinsed with 0.1% PBST, washed 7 times for 5 minutes in 0.1% PBST, twice in sodium citrate for 5 minutes, and 7 times for 5 minutes with dd$H_2O$. After removal of the silicone isolators washed arrays were dried under $N_2$ before scanning.

**2.11 Microarray scanning**

Array scanning was conducted using an Axon Instruments GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA) and GenePix Pro 6.1 software. Slides were scanned immediately upon completion of the hybridization, washing and drying procedures. Scans were performed at 635 nm and 532 nm, the excitation wavelengths of Cy5 and Cy3 respectively. Arrays were scanned at two or three PMT (optical photomultiplier) settings, typically one setting which resulted in 5% saturated pixels, then a lower setting which resulted in no saturation but may leave some spots under-resolved, thus ensuring that results were obtained from all usable spots on each set of arrays. GenePix output includes multiple images and exported data which was subsequently used for data analysis. Scanned images of each slide at each array setting were carefully checked for saturated pixels, missing or malformed spots, scratches, debris and background inconsistencies that might affect the spot values (Figure 2.1). The fluorescence measurements from the dye-labeled protein, caspase 3 antibody and print buffer spots were removed from the scanning file.

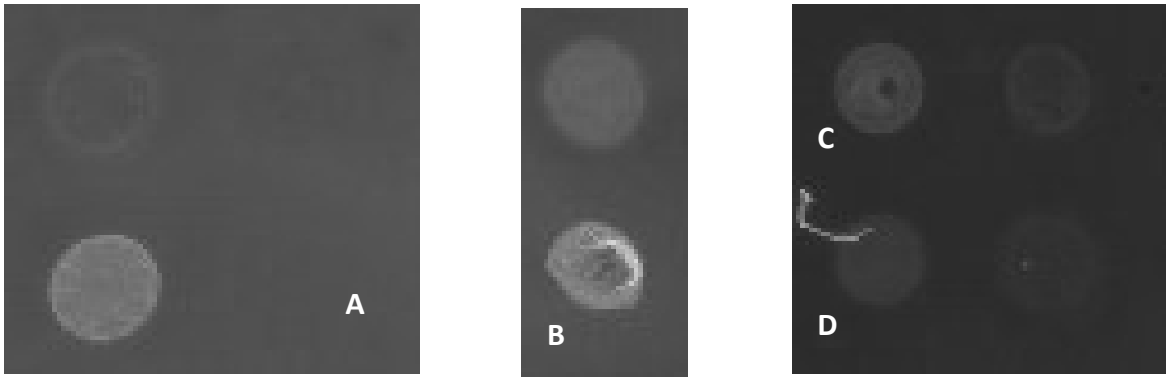


**Figure 2.1** Visual inspection of arrays: missing spots (A), deviant from circular (B), hollow centers (C) and debris contamination (D).

## 2.12 Microarray data processing

Record was kept of the block, column and row of each saturated or defective spot. The dye-labeled protein was intended to be a landmark spot on each array with a constant fluorescence; however no visible fluorescence was detected on any chip. The caspase 3 antibody, intended to be the spike control of the array, was specifically chosen because it did not cross-react with bear skin when tested by Western blotting. Unfortunately, no reaction to purified human caspase 3 protein (sc-1225, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), labeled with Cy3 and Cy5 dyes using the same procedure as described earlier, could be obtained, even with significant addition of the protein. The print buffer spot was designed to be a negative control spot, but spot inconsistencies did not allow its use.

Starting with the text file of scanned data corresponding to the highest laser settings used, over saturation was corrected first. As mentioned earlier, each antibody was spotted once on each of the three blocks that made up the array. If even one spot of the three on the array was over saturated then the set of spots corresponding to one antibody was replaced with the values from a lower laser scan which did not have oversaturated pixels. Next, using the notes made from the visual inspection, each spot that was missing, deviated from circular, had a hollow center, had debris that covered part of the spot, or had been scratched was flagged as a bad spot and not used in any further analysis. This file was then renamed with the slide number and sample name. A revised version of the file was then made by deleting all columns except, Flags, Block, Column, Row, Name, F635 Mean and F532 Mean. The flag column consisted of negative numbers corresponding to spots that were flagged during the visual inspection in the GenePix program. Block, column and row columns identified where the spot was located on the array. Name column identified the antibody that was printed for each spot. F635 mean and F532 mean were the mean values for all the pixels within the spot for the scan at 635nm and 532nm respectively. Using the flag column, all spots flagged as bad were deleted. A column was inserted which calculated the F635/F532 mean for each spot. Columns were then inserted which calculated the average of the three spots corresponding to each antibody, the standard deviation and the coefficient of variation for F635, F532 and F635/F532. Any antibody with a coefficient of variation (standard deviation/mean × 100%) exceeding 15% for F635, F532 or F635/F532 was checked for obvious errors in the replicates. If the deletion of one spot allowed the coefficient of

variation to drop to or below 15% then the other spots were kept. If not then all three spots were deleted. A further spreadsheet was then made containing only the Name and F635/F532 Mean columns. This data was then imported into a larger spreadsheet with all the samples present.

To summarize, scanned values of each of 31 stress-associated proteins from each individual grizzly bear sample run in triplicate on the microarray was standardized by dividing by the value obtained from the pooled grizzly bear reference standard run on the same array. Thus, each grizzly bear sample produced triplicate values for the expression of each stress-associated protein in relation to the same standard sample. These triplicate values were averaged to provide a single "relative protein expression" value to be used for statistical analyses.

**2.13 Intra-array variation**

Ten individual bears were run on separate microarrays and the coefficient of variation was calculated to determine intra-assay variability. This assessment was done after the visual evaluation for missing or malformed spots that were removed.

**2.14 Inter-array variation**

A series of preliminary laboratory validation experiments were conducted using the six bulk tissue samples described previously prior to running samples from individual bears biopsied in the field as well as 4 individual bear samples. Ten individual bears were run on two separate microarrays and the coefficient of variation was calculated to determine inter-assay variability. Intra-array variation was controlled at 15% or less by the methods described previously.

**2.15 Dye exchange experiment**

To determine if dye labeling would have an effect on the values of individual proteins, a "dye flip" experiment was performed. Six arrays were run, with one bear sample labeled with Cy5 and an equal amount of protein from the pooled standard labeled with Cy3. The same six bear samples were then labeled with Cy3 and run on six arrays with an equal amount of protein from the pooled standard labeled with Cy5. The results of the protein expression were then compared to determine if the dye used to label the tissue affected the measured protein expression.

## 2.16 Antibody and protein dilution experiments

As described previously a dilution series of an anti-cytokeratin antibody was printed on each array at dilutions of 1:1, 1:5, and 1:25 in print buffer. An analysis was performed to determine what effect the dilution of the antibodies had on the measured relative cytokeratin expression. In a separate experiment, different amounts of protein (80, 20 and 10 μg) were added to the array in order to determine if small protein quantities could be used on the chips while still receiving a strong signal.

## 2.17 Protein degradation and preservative experiments

The ideal situation when monitoring a cryptic species that covers a vast portion of the landscape is to be able to use incidental samples that become available periodically, such as management kills, self-defense kills and recent road or train kills. Unfortunately, immediate refrigeration or freezing is not possible in these situations. To determine what effect time at room temperature would have on the protein expression, larger skin samples from three bears were sub-sectioned and left at room temperature (22-26$^{o}$C) for 0, 4, 8, 12, 24 or 48 hours. Each sample was placed on a weigh boat covered with parafilm. At the end of the time at room temperature the samples were refrozen and then processed in the same manner as the other samples.

RNA-later (Applied Biosystems / Ambion, Foster City, CA, USA) is well known as a preservative that retards RNA degradation. It is being used by other researchers involved in the FRI Grizzly Bear project and the question arose whether samples immersed in RNA-later could be used for protein determination by microarray. To determine the effects of RNA-later on protein expression and determine if it could slow protein degradation at room temperature, larger skin samples from three bears were sub-sectioned, immersed in a cryovial filled with 400 μl of RNA-later and left for 0, 24, 72 or 336 hours. Before refreezing, each sample was removed from the RNA-later and allowed to drip dry before placing it in a new cryovial.

## 2.18 Assessment of the effects of biological factors and capture method

Grizzly bear sex, age, region of capture, reproductive class and capture method were used as variables to determine if they affected the expression of proteins evaluated by the microarray.

Reproductive classes were defined as solitary adult female ≥4 years old (1), adult female with dependent offspring (2), juvenile female <4 years old (3), adult male (4) and juvenile male <5 years old (5). Capture and health data were provided by Karen Graham, Gordon Stenhouse and Marc Cattet. Regions where the bears were captured are depicted in Figure 2.2. Contributing to the delineation of the regions was the partial population separation caused by the east-west highways discovered by genetic survey (Proctor and Paetkau 2004).

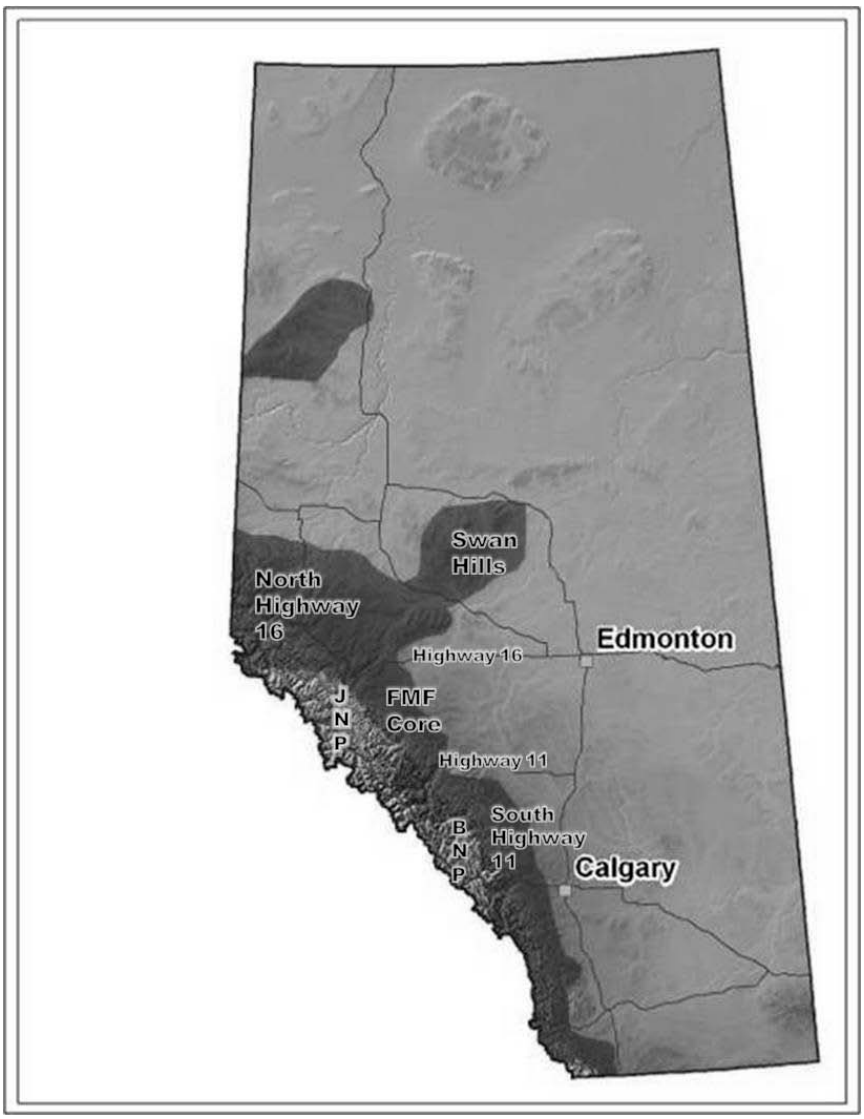


**Figure 2.2** Map of Alberta, Canada with the areas where grizzly bears were captured. Dark shaded areas are areas with known bear populations including the Foothills Model Forest (FMF) Core. The lighter mountain areas in the Southwest are national parks: Jasper National Park (JNP) and Banff National Park (BNF).

### 2.19 Serum measures

Serum-based stress data were provided by Jason Hamilton, Brian Chow, Matt Vijayan and Marc Cattet. Serum total cortisol was measured by a commercial $^{125}$I cortisol radioimmunoassay kit (#07-221102 MP Biomedicals, Irvine, CA, USA). Serum hsp60 and hsp70 were measured using enzyme-linked immunosorbent assay kits (#EKS-600, #EKS-700 Stressgen Biotechnologies, Victoria, BC, Canada) (Hamilton 2007).

### 2.20 Environmental measures

Environmental measurement scores were calculated by Jerome Cranston. They condensed many of the environmental measures taken of 95% of the kernel home range of the grizzlies. The proportion protected score was the proportion of area within a bear's home range that was park or protected land. The road density score was calculated using spatial analyst neighborhood statistics based on circular search of road density within a 1600m radius in the bear's home range. The average of three seasonal resource selection functions (RSF) calculated for a bear's home range was termed the mean RSF. Resource selection functions are calculated by multiple regression of bear use vs. availability of land to model the bear's use of a landscape (Alberta Grizzly Bear Recovery Team 2008). Anthropogenic change was calculated as the proportion of a bear's home range occupied by recently built features. Recently built features were defined as the features built from the fall previous to the year of capture to the fall of the year of capture. Some home ranges had no recently built features.

### 2.21 Allostatic load

Using a modification of Seeman et al. (1997), allostatic load scores were calculated for each bear. For each of the 31 stress proteins, the top quartile of expression among all n=108 bears analyzed with the microarray was determined. For each protein with an expression value within the top quartile a point (1) was assigned. The allostatic load points from the individual protein measures were then summed for each bear to determine that bear's stress protein index. Sex, age, region of capture, reproductive class and capture method were evaluated to determine if they affected the stress protein index of the bears.

### 2.22 Comparison of skin and muscle

Initially the project evaluated muscle to determine stress protein expression, however concern was raised because of the difficulty in obtaining consistently sized samples and because of the length of the wound tract created in the bear's muscle by the biopsy needle. It was decided to switch to skin as sampling is less invasive and incidental samples are taken on any new bear that is captured (bears that have not been previously captured have small discs of skin removed from the ear to allow for the placement of identifying ear tags). A comparison of the protein expression in skin and muscle samples was performed.

### 2.23 Comparison of skin sampling locations

To assess whether the location on the body had an impact on the protein expression found in the skin, multiple locations on the body were sampled (ear plug, neck, fore leg and hind leg).

### 2.24 Statistical analyses

The statistical software SAS 9.1 was used to perform the statistical analyses of normality and correlations (SAS Institute Inc., Cary, NC, USA). Normality, skewness and kurtosis of the data were tested (Zar 1999). Normality was also assessed with boxplots, Kolmogorov-Smirnov test and normal probability plots. Deviations from normality in some variables were observed, so log10 transformation was performed on body measures of length and mass as well as the serum measures of GGT, total cortisol, hsp 60 and hsp70. The coefficient of variation (%CV; SD/mean x 100%) was used to assess the intra-array and inter-assay variation. Pearson correlation was performed to evaluate relationships between proteins in bears. The remaining statistical analyses were performed using statistical software SPSS 19 (SPSS Inc., Chicago, IL, USA).

For the comparison of tissue types (skin and muscle), skin sampling location, dye exchange experiment, differing antibody and protein concentrations, protein degradation and preservative effects analyses the larger tissue samples from six bears were used. Microarray validation analysis of dye effects, muscle vs. skin comparison, skin sampling location comparison, protein quantity comparison, antibody dilution, and decay evaluation were performed with repeated measures ANOVA, type IV model, followed by Sidak post-hoc

comparison where appropriate. Analysis of the effects of RNA-later was performed with paired t-tests comparing preserved and unpreserved samples at 0 and 24 hours. For all comparisons proteins were evaluated as a group by protein category (see Table 2.2). A Bonferroni correction was performed to correct for the 4 protein categories being evaluated ($p<0.013$). Missing values, eliminated from analysis during preprocessing, were interpolated prior to analysis by averaging protein values within the protein category and treatment.

First principal component values were calculated for each protein category by using principal component analysis (PCA) for dimension reduction of all proteins within the category based on eigenvalues >1. Stress protein index was calculated by assigning a point to each bear for each protein expression at or above the $75^{th}$ percentile in expression for that protein and summing the points for each bear.

Investigative modeling with univariate Type IV model ANOVA was performed to evaluate whether biological, capture, growth, serum measures or environmental measures were related to the protein category expression as measured by first principal component or stress protein differential score. Only the capture methods of culvert trap, helicopter dart and snare were included in the analysis as the capture methods hunter kill, NWT Wildlife service, hit by vehicle and zoo provided insufficient (1-2) bears captured by these methods. Biological measures were evaluated first and included region, sex, age, year captured, Julian day captured and an interaction between capture year and region. The interaction between capture year and region was included as trapping effort in specific regions differed by year. Once a biological model was found with all variables significant ($p<0.1$), capture variables were added to the model. Capture variables included capture method, number of times each bear was captured and an interaction between region and capture method. The interaction between region and capture method was included as terrain and accessibility differences resulted in capture method differences between regions. The biological and capture model with only significant variables ($p<0.1$) was then modified with the addition of growth variables. Growth variables included log length, log mass and BCI. The significant biological, capture and growth model was then modified with serum measures. Serum measures included log GGT, log total cortisol, log hsp60 and log hsp70. The best model was then chosen and accepted as the model for that protein category with high sample size. Another model was also created using the best model plus

environmental measures. Environmental measures included anthropogenic change, mean RSF, road density and proportion protected. The best model with these variables was also determined. The model including the environmental measures had a reduced sample size as environmental measures were not determined for all bears. This reduced sample size is the reason two models were selected as it was feared that some variables in the environmental model would be eliminated solely due to the smaller sample size and not because they were not related to the protein category. Both final models for each protein categories were further evaluated using Sidak post-hoc testing.

## Chapter 3 Results

### 3.1 Two dimensional polyacrylamide gel electrophoresis

Two dimensional gel electrophoresis evaluations revealed several proteins that were elevated or depressed in the muscle and skin from 2 bears matched by age and sex with contrasting body conditions and presumed to be differentially stressed (Tables 3.1 and 3.2). In muscle, higher expression of ATP synthase subunits β, e and complex F1, mitochondrial succinate dehydrogenase flavoprotein subunit, hemoglobin α, succinyl-CoA synthase β subunit, myosin light chain 1, α-actin, annexin A5, stratifin, malate dehydrogenase, and porin 31HM was observed in the bear believed to be more stressed. In contrast, hsp27, ATP synthase F0 complex, substrate protein of mitochondrial ATP-dependent proteinase SP-22, electron transfer flavoprotein, α B-crystallin, glyceraldehyde 3-phosphate dehydrogenase, enolase 3, desmin, troponin, tropomyosin, triosephosphate isomerase, myoglobin, myosin light chain 1, actin, ubiquinol-cytochrome c reductase, tripartite motif protein 50, peroxiredoxin 6 and α tropomyosin had lower expression in bears believed to more stressed. In skin, higher expression of hemoglobin α and β, keratin 1 and stratifin, and lower expression of albumin, β-actin, myoglobin, aldehyde dehydrogenase, immunoglobulin precursor and transferrin, were observed in the bear believed to be more stressed. Although certain of these proteins represented good candidates for stress-associated proteins to include in the microarray, commercial antibodies that recognize them in grizzly bear skin were not found, with the exception of hsp27.

**Table 3.1** Proteins with elevated expression when comparing a bear thought to be more stressed to a matched bear presumed to be less stressed.

| **Protein identity** | **Tissue** | **Year** |
|---|---|---|
| α-actin | Muscle | 2007 |
| annexin A5 | Muscle | 2007 |
| ATP synthase β subunit | Muscle | 2007 |
| ATP synthase, h+ transporting, mitochondrial f1f0 complex, subunit e | Muscle | 2006 |
| ATP-specific succinyl-CoA synthase β subunit | Muscle | 2007 |
| hemoglobin α | Muscle | 2006 |
| mitochondrial ATP synthase H+ transporting F1 complex α | Muscle | 2007 |
| porin 31HM | Muscle | 2007 |
| Predicted: similar to malate dehydrogenase, cytoplasmic isoform 1 | Muscle | 2007 |
| succinate dehydrogenase (ubiquinone) flavoprotein subunit, mitochondria | Muscle | 2007 |
| Predicted: similar to fatty-acid binding protein adipocyte | Skin | 2006 |
| hemoglobin α | Skin | 2006 |
| hemoglobin β | Skin | 2006 |
| Predicted: similar to keratin 1, isoform 1 | Skin | 2006 |
| stratifin | Skin | 2007 |

**Table 3.2** Proteins with lower expression when comparing a bear thought to be more stressed to a matched bear presumed to be less stressed.

| **Protein identity** | **Tissue** | **Year** |
|---|---|---|
| α B-crystallin | Muscle | 2007 |
| ATP synthase, H+ transporting, mitochondrial F0 complex | Muscle | 2007 |
| desmin | Muscle | 2007 |
| electron transfer flavoprotein, α peptide | Muscle | 2006 |
| enolase 3 | Muscle | 2007 |
| fast muscle actin | Muscle | 2007 |
| glyceraldehyde-3-phosphate dehydrogenase | Muscle | 2007 |
| heat shock protein 27 | Muscle | 2006 and2007 |
| myoglobin | Muscle | 2007 |
| Predicted: peroxiredoxin 6 | Muscle | 2007 |
| Predicted: similar to tripartite motif protein 50 | Muscle | 2007 |
| Predicted: similar to ubiquinol-cytochrome c reductase core protein | Muscle | 2007 |
| striated muscle α tropomyosin | Muscle | 2006 |
| substrate protein of mitochondrial ATP-dependent proteinase SP-22 | Muscle | 2007 |
| triosephosphate isomerase (TIM) (Triose-phosphate isomerase) | Muscle | 2007 |
| tropomyosin, α isoform | Muscle | 2006 |
| tropomyosin, chain b | Muscle | 2006 |
| troponin | Muscle | 2007 |
| albumin | Skin | 2007 |
| aldehyde dehydrogenase, mitochondrial (ALDH class 2) | Skin | 2007 |
| β-actin | Skin | 2007 |
| myoglobin | Skin | 2007 |
| Predicted: similar to Immunoglobulin lambda-like polypeptide 1 precursor | Skin | 2007 |
| transferrin | Skin | 2007 |

### 3.2 Commercial microarrays

Very few of the antibodies from the three commercial protein microarrays tested recognized grizzly bear proteins. Although initial scanning of the commercial microarrays revealed putative signals from bear skin, further validation and confirmation attempts using western blotting showed that only 7 of 58 antibodies against stress-associated proteins cross-reacted with grizzly bear proteins (Appendix A1.1-A1.3). Six antibodies were from the Sigma Panorama cell signaling array: caspase 6 (C7599), iNOS (N7782), MAP kinase (M7927), MAP kinase p38 (M0800), protein kinase Bα (PKBα) (P1601) and PRDX3 (P1247), and one from the Spring Bioscience array (myoglobin; E2994). Of these 7 antibodies, two were selected for use in our custom microarray: caspase 6 and iNOS from the Sigma Panorama array. Although the lack of useful information from the commercial microarrays was discouraging, it reinforced the need to develop a custom grizzly bear-specific protein microarray.

### 3.3 Grizzly bear protein microarray

#### 3.3.1 Array validation

##### 3.3.1.1 Intra- and inter-assay variation

To determine the consistency of the results obtained from the 3 individual spots on each array, intra-assay repeatability using the %CV was calculated for each protein. Ten individual bear samples were run on separate microarrays, and the mean %CV between the 3 spots on each array was calculated (Table 3.3). This calculation was done after the visual evaluation of spot quality was used to eliminate some spots. The majority of proteins (28/31) had a %CV below 10 percent, and three proteins had a %CV between 10 to 15 percent.

To determine the reproducibility of results obtained from the same samples run on separate microarrays, inter-assay repeatability using %CV was calculated for each protein. Ten individual bear samples were run on two separate arrays, and the mean %CV between the two arrays was calculated (Table 3.3). The majority of proteins (27/31) had a %CV below 15 percent, and four proteins had a %CV between 15 to 18 percent.

The dye labelled spot (internal control), print buffer spot (negative control) and caspase 3 spot (positive control) with purified caspase 3 could not be used to normalize between the arrays.

The dye labelled spot added by the print company did not fluoresce, the print buffer spots were inconsistent and the caspase 3 antibody did not react to the purified sample of caspase 3 added. The pooled sample labelled with Cy3 run on each array was used as an internal control.

**Table 3.3** Intra- and inter- array variation: coefficient of variation (%CV) assessment of protein expression measurement. Abbreviations for proteins are found on pp. x-xi.

| Protein | Intra-array variation (mean, n) | Inter-array variation (mean, n) |
|---|---|---|
| ACTH | 4.27, 10 | 8.9, 9 |
| AVP R V1a | 6.82, 10 | 9.4, 9 |
| CRH-R 1/2 | 8.18, 10 | 10, 10 |
| GR | 6.46, 10 | 10.5, 10 |
| POMC | 11.90, 10 | 17.6, 8 |
| Prolactin | 5.42, 10 | 10.1, 10 |
| AIF | 7.84, 10 | 9.1, 9 |
| Annexin II | 8.93, 10 | 6.4, 10 |
| Annexin IV | 9.17, 10 | 7, 10 |
| Caspase 1 | 7.67, 10 | 11.6 |
| Caspase 2 | 6.58, 10 | 6.5 |
| Caspase 6 | 4.76, 4 | 12.3 |
| E cadherin | 6.87, 10 | 9.1 |
| GAPDH | 7.84, 10 | 15.4 |
| Cytokeratin | 4.86, 10 | 10.8 |
| Grp78 | 2.42, 9 | 12.9, 7 |
| Hsp27 | 2.87, 6 | 12, 4 |
| Hsp40 | 5.63, 10 | 8.2, 10 |
| Hsp60 | 3.81, 2 | 13.8, 6 |
| Hsp70 | 7.60, 9 | 6.8, 9 |
| Hsp70i | 11.26, 10 | 8.9, 10 |
| Hsp90 | 1.83, 5 | 15.8, 6 |
| Hsp110 | 4.59, 8 | 6.8, 9 |
| CCR5 | 10.26, 10 | 10.7, 9 |
| COX2 | 7.51, 10 | 7, 10 |
| HO2 | 8.03, 10 | 7.2, 10 |
| eNOS | 4.92, 8 | 15.8, 10 |
| iNOS | 2.38, 5 | 6.3, 3 |
| PRDX3 | 3.88, 10 | 7.9, 9 |
| SOD1 | 6.98, 10 | 9.7, 10 |
| SOD2 | 5.22, 10 | 5.6, 9 |

### 3.3.1.2 Dye experiment

The effect of switching the fluorescent dye used to label the samples vs. the pooled standard was investigated for each protein category. Matched samples analyzed on the array in two ways, where the sample was first labelled with Cy5 and the pooled sample was labelled with Cy3 and the second run the labelling was reversed (sample with Cy3 and pooled standard with Cy5). Protein expression measured for bear samples labeled with Cy5 was elevated compared to the same bear samples labeled with Cy3 for HPA proteins (repeated measures ANOVA; $F=19.0$, $p<0.001$, $n=6$), ACC proteins (repeated measures ANOVA; $F=51.5$, $p<0.001$, $n=6$), CS proteins (repeated measures ANOVA; $F=11.4$, $p=0.001$, $n=6$) and OSI proteins (repeated measures ANOVA; $F=42.8$, $p<0.001$, $n=6$). Significant ANOVAs were followed up by Sidak post-hoc tests (HPA proteins ($p<0.001$), ACC proteins ($p<0.001$), CS proteins ($p=0.001$), OSI proteins ($p<0.001$)) (Figure 3.1).

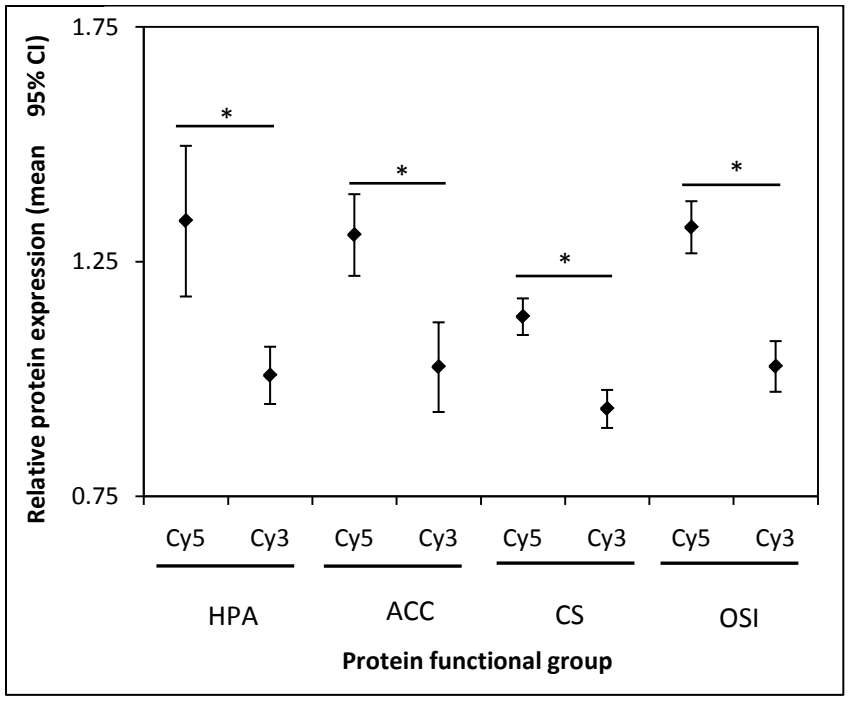


**Figure 3.1** Effects of switching fluorescent dye labels (Cy5 to Cy3) on measured grizzly bear skin protein expression. Mean values labeled with asterisks are significantly different ($p<0.0125$) from each other, as determined by repeated measures ANOVA followed by Sidak test ($n=6$). Abbreviations: ACC, apoptosis and cell cycle, CI, confidence interval; CS, cellular

stress; Cy, cyanine; HPA, hypothalamic-pituitary-adrenal axis, OSI, oxidative stress and inflammation.

### 3.3.1.3 Antibody dilution

To determine the effect of diluting the antibody with increasing amounts of printing buffer before printing microarray slides, three spots were added to the array with differing dilutions of the cytokeratin antibody. This antibody dilution was one of the inter-assay controls included in each microarray. A difference was detected between the cytokeratin antibody dilutions (Figure 3.2). Increasing dilution of cytokeratin antibody with printing buffer had a significant effect on measured cytokeratin expression (repeated measures ANOVA, $F=384.5$, $p<0.001$, $n=86$). Each antibody dilution was significantly different from each other and there was decreasing cytokeratin expression with increasing dilution (Sidak, $p<0.0001$). Inconsistencies in spot morphology were observed with increasing amounts of printing buffer (Figure 3.3).

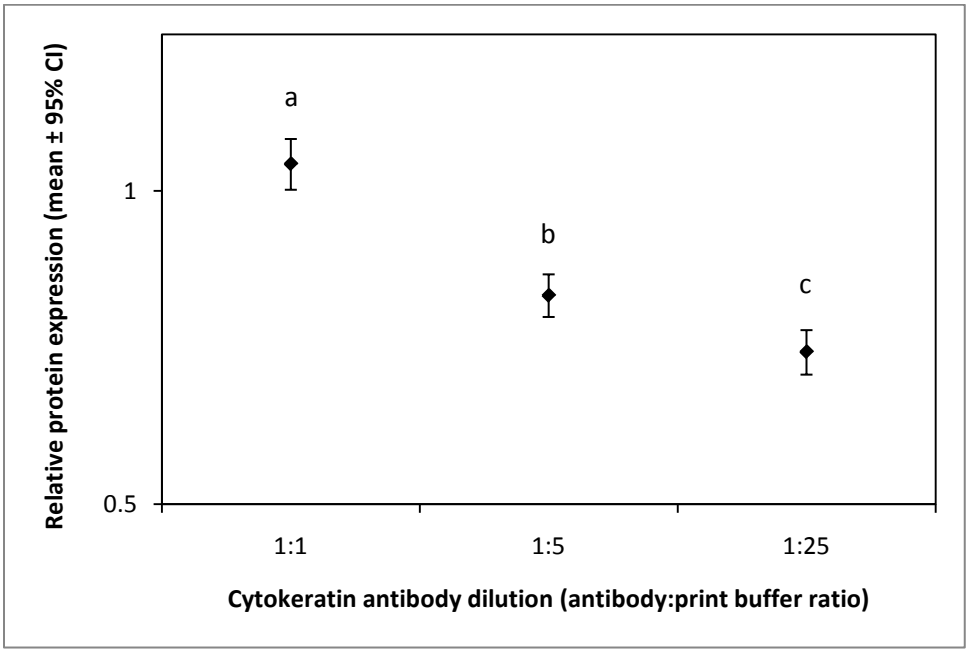


**Figure 3.2** Effects of cytokeratin antibody dilution on measured cytokeratin protein expression in grizzly bear skin. The cytokeratin antibody dilution was 1 part antibody to 1, 5 or 25 parts printing buffer. Significant differences were found with a>b>c, as determined by repeated measures ANOVA followed by Sidak test ($p<0.0125$).

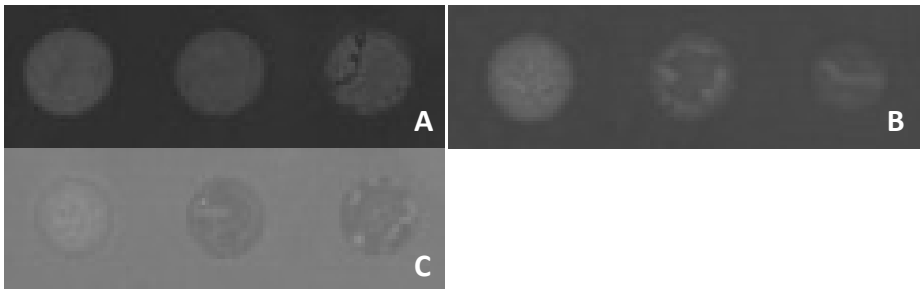


**Figure 3.3** Printing inconsistencies detected with increasing printing buffer concentration. Spots were printed from left to right as 1:1, 1:5 and 1:25 dilutions of cytokeratin antibody: printing buffer. **A**: black lines in the 1:25 spots. **B**: Reduced size of the 1:25 spots and inconsistent spot morphology. **C**: Missing centers, especially 1:25 spots.

### 3.3.1.4 Comparison of protein quantities

To determine if very small skin samples with low protein yields could be run and still produce a detectable signal on the antibody based array, a protein dilution series was run. The protein dilution series consisted of different protein quantities (10, 20 and 80 µg) from three individual bears paired with the pooled standard at the same protein quantity. The protein quantity was found to have a significant effect on measured HPA protein expression (repeated measures ANOVA, $F=67.5$, $p<0.001$, $n=2$), ACC protein expression (repeated measures ANOVA, $F=68.6$, $p<0.001$, $n=2$), CS protein expression (repeated measures ANOVA, $F=13.3$, $p<0.001$, $n=2$) and OSI protein expression (repeated measures ANOVA, $F=33.8$, $p<0.0001$, $n=2$). Protein quantity was found to have an effect on the measured protein expression (Sidak post-hoc test, $p<0.0125$), and an increasing measure of protein expression with increasing protein amount was detected (Figure 3.4). The CS proteins measured at 10 µg versus 20µg were not significantly different (Sidak, $p=0.013$), but the trend was continued.

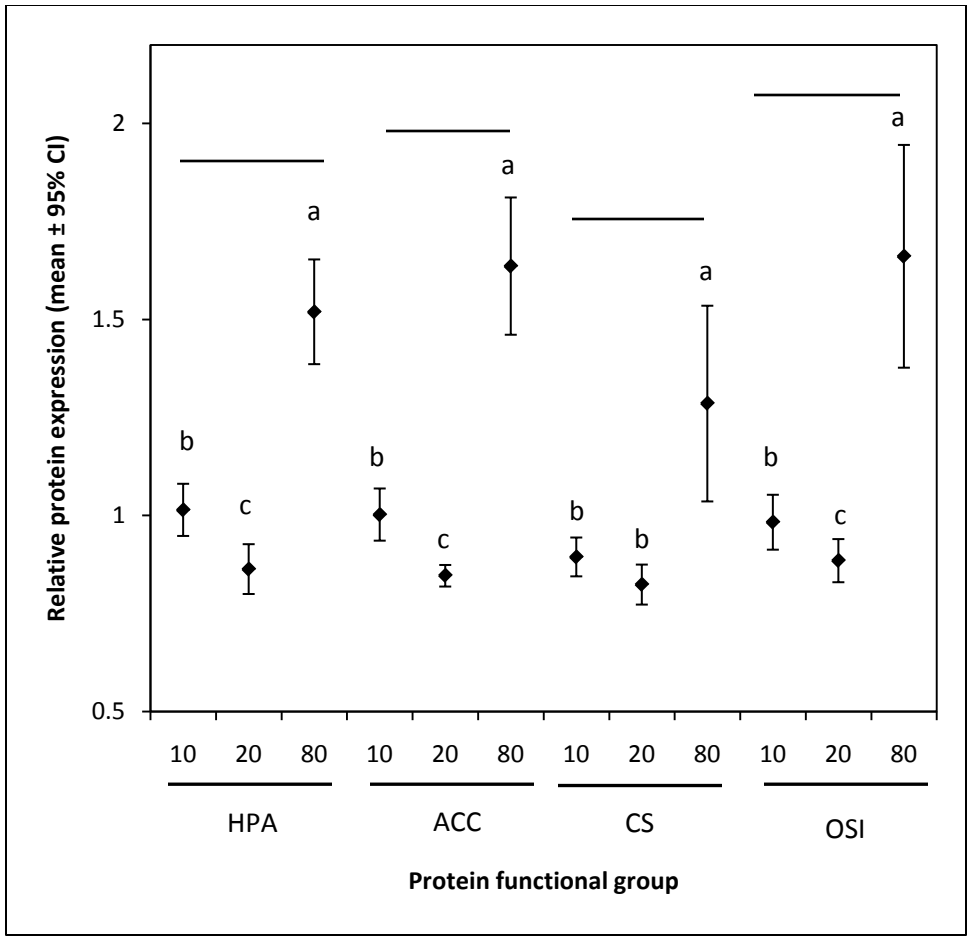


**Figure 3.4** Effects of differing protein amounts on measured protein expression in grizzly bear skin. Significant differences ($p<0.0125$) were found as determined by repeated measures ANOVA followed by Sidak test (n=2). Results of comparison of means by Sidak test are indicated by lower case letters where: a>b>c. Abbreviations: ACC, apoptosis and cell cycle, CI, confidence interval; CS, cellular stress; HPA, hypothalamic-pituitary-adrenal axis, OSI, oxidative stress and inflammation.

### 3.3.1.5 Protein degradation

To determine if skin samples that had undergone some level of protein degradation, as might occur in field collections, could be used for protein microarray analysis, an experiment was conducted in which sub-sectioned grizzly bear skin from three individual bears was subjected to varying amounts of time (4 to 48 hours) at room temperature. The time at room temperature was found to have a significant effect on measured HPA protein expression (repeated measures ANOVA, $F=21.2$, $p<0.001$, n=3), ACC protein expression (repeated

measures ANOVA, F=11.2, p<0.001, n=3), CS protein expression (repeated measures ANOVA, F=6.9, p<0.001, n=3) and OSI protein expression (repeated measures ANOVA, F=4.8, p=0.001, n=3). Lower measured protein expression with increasing time at room temperature was detected for HPA proteins and ACC proteins (Sidak post-hoc test, p<0.013) (Figure 3.5). The trend of decreased measured protein was also found as a trend for zero hours compared to 48 hours (Sidak, p=0.049). The measured expression of CS proteins was higher at 48 hours compared to 24 hours. No significant differences were found for OSI proteins, however a trend for increased expression measured at zero hours and twelve hours compared to 24 hours was detected (Sidak, p=0.031 and 0.019 respectively).

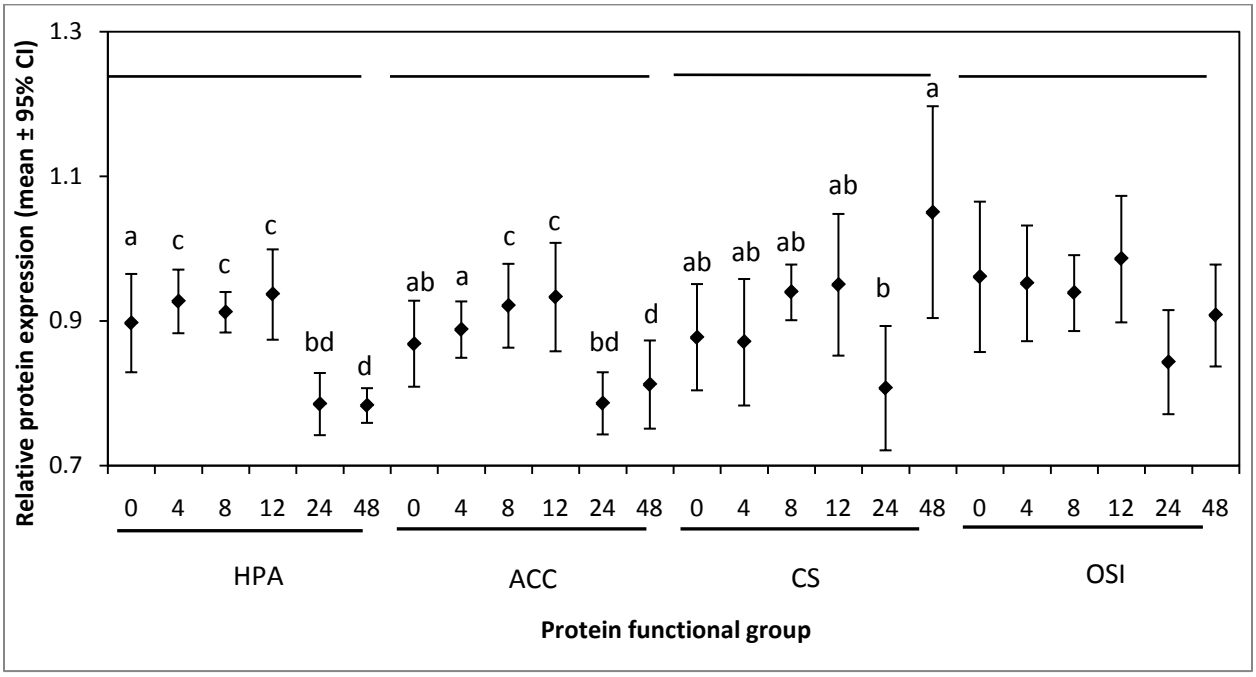


**Figure 3.5** Effects of holding grizzly bear skin samples at room temperature for 4 to 48 hours on expression of microarray proteins. Significant differences (p<0.0125) were found as determined by repeated measures ANOVA followed by Sidak test (n=3). Results of comparison of means by Sidak test are indicated by lower case letters where: a>b>c>d. Abbreviations: ACC, apoptosis and cell cycle, CI, confidence interval; CS, cellular stress; HPA, hypothalamic-pituitary-adrenal axis, OSI, oxidative stress and inflammation.

### 3.3.1.6 Comparison of protein degradation with and without preservative

To determine what effect the commonly used preservative RNA-later, in comparison to no preservative, might have on samples that had undergone potential protein degradation, an experiment was conducted in which sub-sectioned grizzly bear skin from three individual bears was subjected to 24 hours at room temperature either immersed in RNA-later or unpreserved. A significant difference was observed between preserved and unpreserved samples at zero hours at room temperature for HPA proteins (paired t test; $t=-4.8$, $p<0.001$, $n=3$). No difference was observed between preserved and unpreserved samples at zero hours at room temperature for ACC proteins (paired t test; $t=-2.7$, $p=0.013$, $n=3$), CS proteins (paired t test; $t=0.2$, $p=0.85$, $n=3$) and OSI proteins (paired t test; $t=0.2$, $p=0.84$, $n=3$). No difference was observed between preserved and unpreserved samples at 24 hours at room temperature for HPA proteins (paired t test; $t=-2.2$, $p=0.05$, $n=2$), ACC proteins (paired t test; $t=-2.2$, $p=0.043$, $n=2$), CS proteins (paired t test; $t=-2.7$, $p=0.016$, $n=2$) and OSI proteins (paired t test; $t=-2.1$, $p=0.053$, $n=2$) (Figure 3.6). Although not significant, there was a trend for higher protein expression in the samples preserved with RNA-later within all protein categories after 24 hours at room temperature compared to unpreserved samples.

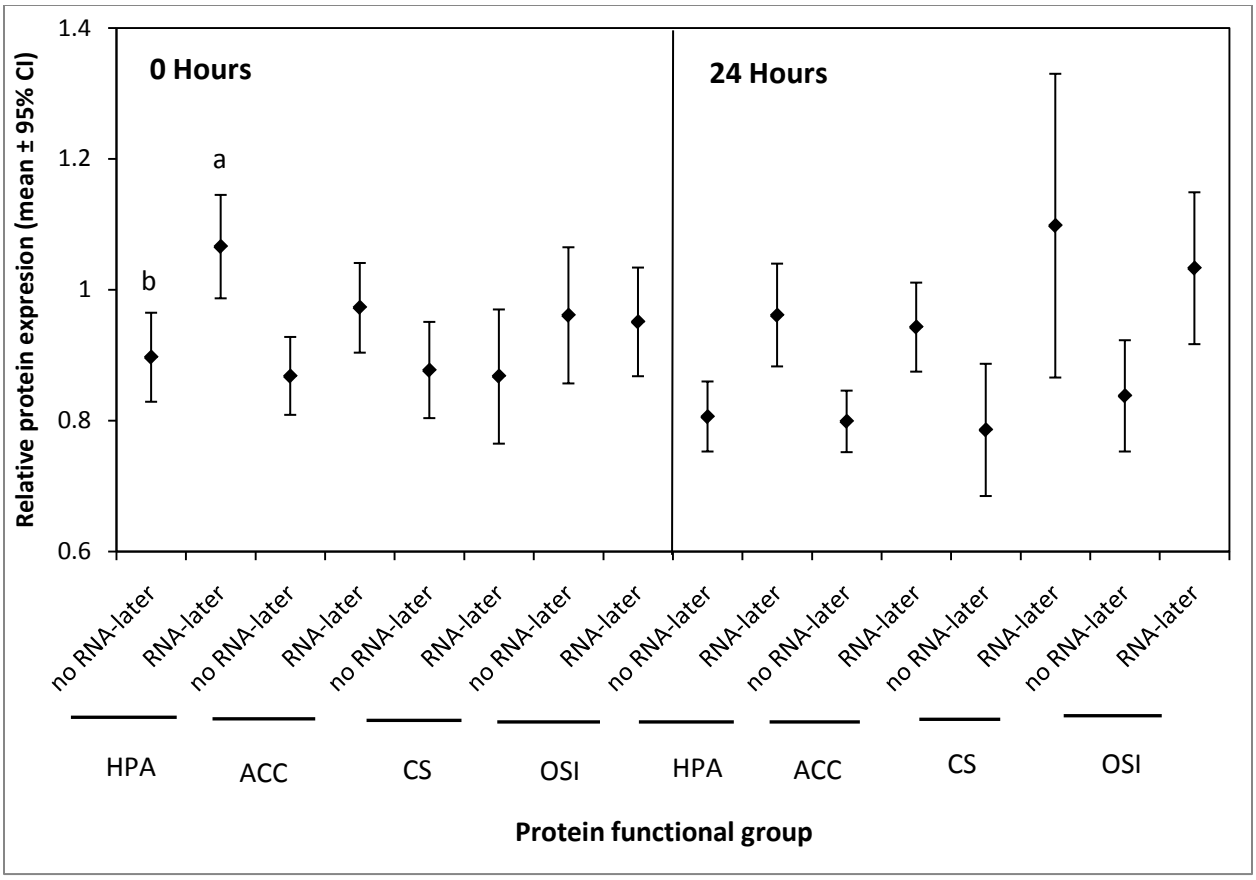


**Figure 3.6** Comparison of measured expression of protein function groups between grizzly bear skin samples preserved with RNA-later with unpreserved samples after holding the samples for 0 to 24 hours at room temperature. Significant differences ($p<0.0125$) were found as determined by paired t test ($n=3$). Results of comparison of means test are indicated by lower case letters where: a>b. Abbreviations: ACC, apoptosis and cell cycle, CI, confidence interval; CS, cellular stress; HPA, hypothalamic-pituitary-adrenal axis, OSI, oxidative stress and inflammation.

### 3.3.1.7 Tissue comparison (skin and muscle)

Samples from muscle and skin tissue were evaluated to determine if there were differences in protein expression. A difference in protein expression between skin and muscle was detected for HPA proteins (repeated measures ANOVA; $F=130.2$, $p<0.001$, $n=3$), ACC proteins (repeated measures ANOVA; $F=324.6$, $p<0.001$, $n=3$), CS proteins (repeated measures ANOVA; $F=21.8$, $p<0.001$, $n=3$) and OSI proteins (repeated measures ANOVA; $F=121.6$, $p<0.001$, $n=3$). For all protein categories, elevated protein expression in skin compared to

muscle samples was confirmed by Sidak test (HPA proteins ($p<0.001$), ACC proteins ($p<0.001$), CS proteins ($p<0.001$) and OSI ($p<0.001$)) (Figure 3.7). As discussed in the Methods, as this microarray was being developed it was decided to focus on grizzly bear skin samples as they were less invasive to obtain and could be incidentally collected when placing tags in bear ears for identification.

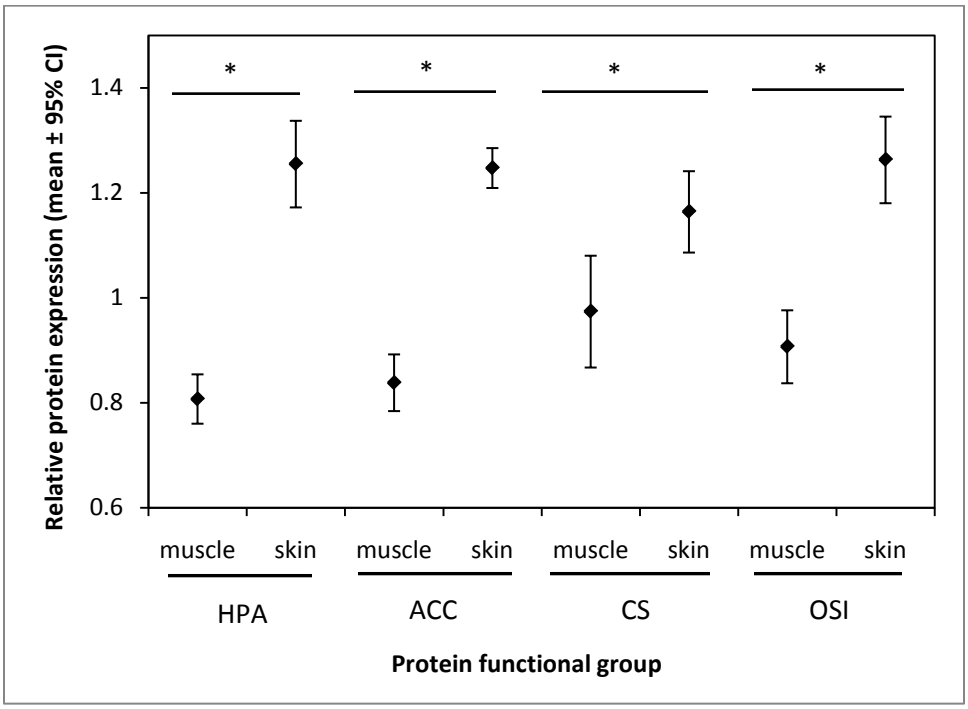


**Figure 3.7** Comparison of protein expression in grizzly bear skin and muscle. Skin was found to have higher mean expression ($p<0.0125$, marked with asterisks) as determined by repeated measures ANOVA followed by Sidak test ($n=3$). Abbreviations: ACC, apoptosis and cell cycle, CI, confidence interval; CS, cellular stress; HPA, hypothalamic-pituitary-adrenal axis, OSI, oxidative stress and inflammation.

### 3.3.1.8 Skin sampling location comparison

A detailed experiment was conducted to investigate the effect of skin sample location (ear plug, neck, fore leg [triceps] and hind leg [thigh]) on protein expression. Only a limited number of bears were sampled for multiple tissues, because of concern about extended anaesthesia time and creating multiple small wounds. Samples from differing skin locations were evaluated to determine if there were differences in protein expression. A protein

expression difference between skin locations was not detected for HPA proteins (repeated measures ANOVA; F=0.7, p=0.54, n=4), ACC proteins (repeated measures ANOVA; F=0.1, p=0.94, n=4), CS proteins (repeated measures ANOVA; F=2.4, p=0.08, n=4) and OSI proteins (repeated measures ANOVA; F=1.1, p=0.34, n=4) (Figure 3.8).

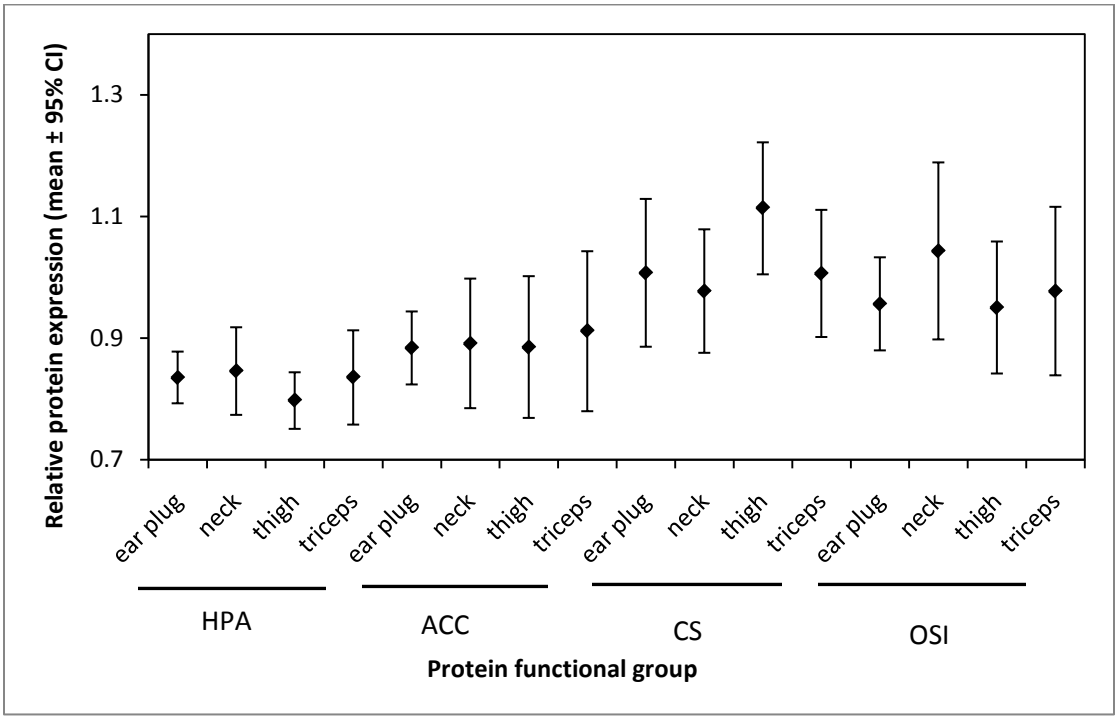


**Figure 3.8** Comparison of protein expression sampled from different skin locations. Abbreviations: ACC, apoptosis and cell cycle, CI, confidence interval; CS, cellular stress; HPA, hypothalamic-pituitary-adrenal axis, OSI, oxidative stress and inflammation.

### 3.3.2 Correlations between proteins

Initial statistical analyses with the grizzly specific microarray indicated that many proteins were significantly correlated both within and between the four protein categories. Within the HPA axis and apoptosis and cell cycle proteins, all proteins were positively correlated (Table 3.4 and 3.5). Within the cellular stress proteins, 6 of 8 were positively correlated with cytokeratin, grp78 was positively correlated with 1 of 8 proteins, hsp27 was positively correlated with 2 of 8 proteins, hsp60, hsp70i and hsp90 were positively correlated with 3 of 8 proteins, and hsp40, hsp70 and hsp110 were positively correlated with 4 of 8 proteins (Table 3.6). Within the oxidative stress and inflammation proteins, iNOS was positively correlated with 1 of 8 proteins,

eNOS was positively correlated with 5 of 8 proteins and CCR5, COX2, HO2, PRDX3, SOD1 and SOD2 were positively correlated with 6 of 8 proteins (Table 3.7).

**Table 3.4** Correlations among proteins involved in the hypothalamic-pituitary-adrenal axis. Values reported are the r (Pearson correlation coefficient), p-value and the number of bears sampled. Highlighted numbers are significant, as determined by Pearson correlation ($p<0.0016$). Abbreviations for proteins are found on pp. x-xi.

| | ACTH | AVP R V1a | CRH-R 1/2 | GR | POMC | Prolactin |
|---|---|---|---|---|---|---|
| ACTH | | | | | | |
| AVP R V1a | **0.65, <.0001, 104** | | | | | |
| CRH-R 1/2 | **0.75, <.0001, 102** | **0.94, <.0001, 105** | | | | |
| GR | **0.75, <0.0001, 103** | **0.95, <0.0001, 106** | **0.98, <0.0001, 104** | | | |
| POMC | **0.71, <.0001, 100** | **0.68, <.0001, 103** | **0.77, <.0001, 100** | **0.78, <0.0001, 101** | | |
| Prolactin | **0.75, <.0001, 103** | **0.95, <.0001, 107** | **0.96, <.0001, 104** | **0.98, <0.0001, 105** | **0.77, <.0001, 102** | |

**Table 3.5** Correlations among proteins involved in apoptosis and the cell cycle. Values reported are the r (Pearson correlation coefficient), p-value, and the number of bears sampled. Highlighted numbers are significant, as determined by Pearson correlation ($p<0.0016$). Abbreviations for proteins are found on pp. x-xi.

| | AIF | Annexin II | Annexin IV | Caspase 1 | Caspase 2 | Caspase 6 | E-cadherin | GAPDH |
|---|---|---|---|---|---|---|---|---|
| AIF | | | | | | | | |
| Annexin II | **0.93, <.0001, 103** | | | | | | | |
| Annexin IV | **0.95, <.0001, 103** | **0.99, <.0001, 104** | | | | | | |
| Caspase 1 | **0.95, <.0001, 105** | **0.96, <.0001, 104** | **0.97, <.0001, 105** | | | | | |
| Caspase 2 | **0.76, <.0001, 104** | **0.82, <.0001, 103** | **0.82, <.0001, 104** | **0.77, <.0001, 107** | | | | |
| Caspase 6 | **0.64, <.0001, 47** | **0.64, <.0001, 45** | **0.64, <.0001, 45** | **0.62, <.0001, 47** | **0.81, <.0001, 47** | | | |
| E-cadherin | **0.97, <.0001, 104** | **0.97, <.0001, 103** | **0.98, <.0001, 104** | **0.97, <.0001, 106** | **0.81, <.0001, 105** | **0.66, <.0001, 46** | | |
| GAPDH | **0.53, <.0001, 104** | **0.45, <.0001, 103** | **0.46, <.0001, 104** | **0.50, <.0001, 107** | **0.56, <.0001, 106** | **0.55, <.0001, 46** | **0.50, <.0001, 105** | |

**Table 3.6** Correlations among proteins involved in the cellular stress cascade. Values reported are the r (Pearson correlation coefficient), p-value, and the number of bears sampled. Highlighted numbers are significant, as determined by Pearson correlation ($p<0.0016$). Abbreviations for proteins are found on pp. x-xi.

| | Cyto-keratin | Grp78 | Hsp27 | Hsp40 | Hsp60 | Hsp70 | Hsp70i | Hsp90 | Hsp110 |
|---|---|---|---|---|---|---|---|---|---|
| Cyto-keratin | | | | | | | | | |
| Grp78 | 0.06, 0.5447, 93 | | | | | | | | |
| Hsp27 | 0.24, 0.0652, 59 | 0.30, 0.0223, 56 | | | | | | | |
| Hsp40 | **0.55, <.0001, 106** | 0.22, 0.0289, 95 | -0.04, 0.7513, 60 | | | | | | |
| Hsp60 | **0.49, <.0001, 60** | -0.26, 0.0522, 58 | **0.57, 0.0002, 38** | 0.18, 0.1629, 62 | | | | | |
| Hsp70 | **0.50, <.0001, 105** | 0.30, 0.0032, 94 | -0.12, 0.3477, 59 | **0.82, <.0001, 107** | -0.06, 0.6422, 61 | | | | |
| Hsp70i | **0.60, <.0001, 102** | -0.03, 0.7988, 91 | -0.13, 0.3468, 58 | **0.83, <.0001, 104** | 0.20, 0.1304, 60 | **0.75, <.0001, 103** | | | |
| Hsp90 | **0.52, <.0001, 78** | 0.22, 0.0597, 73 | **0.72, <0.0001, 52** | 0.03, 0.8228, 79 | **0.63, <.0001, 53** | 0.05, 0.6333, 78 | 0.02, 0.8314, 76 | | |
| Hsp110 | **0.31, 0.0014, 101** | **0.85, <0.0001, 93** | 0.34, 0.0094, 57 | **0.48, <.0001, 103** | 0.02, 0.8783, 61 | **0.47, <.0001, 102** | 0.26, 0.0081, 99 | 0.25, 0.0304, 77 | |

**Table 3.7** Correlations among proteins involved with oxidative stress and inflammation. Values reported are the r (Pearson correlation coefficient), p-value, and the number of bears sampled. Highlighted numbers are significant, as determined by Pearson correlation ($p<0.0016$). Abbreviations for proteins are found on pp. x-xi.

| | CCR5 | COX2 | HO2 | eNOS | iNOS | PRDX3 | SOD1 | SOD2 |
|---|---|---|---|---|---|---|---|---|
| CCR5 | | | | | | | | |
| COX2 | **0.81, <.0001, 104** | | | | | | | |
| HO2 | **0.78, <0.0001, 102** | **0.96, <0.0001, 106** | | | | | | |
| eNOS | **0.79, <.0001, 95** | **0.72, <.0001, 98** | **0.67, <0.0001, 96** | | | | | |
| iNOS | 0.15, 0.2227, 67 | 0.23, 0.0614, 69 | 0.30, 0.0139, 67 | 0.09, 0.4650, 63 | | | | |
| PRDX3 | **0.32, 0.0012, 102** | **0.38, <.0001, 106** | **0.43, <0.0001, 104** | 0.21, 0.0387, 96 | **0.88, <.0001, 69** | | | |
| SOD1 | **0.78, <.0001, 104** | **0.97, <.0001, 108** | **0.97, <0.0001, 106** | **0.72, <.0001, 98** | 0.24, 0.0509, 69 | **0.42, <.0001, 106** | | |
| SOD2 | **0.69, <.0001, 98** | **0.72, <.0001, 102** | **0.72, <.0001, 101** | **0.55, <.0001, 93** | 0.12, 0.3511, 65 | **0.34, 0.0005, 100** | **0.76, <.0001, 102** | |

### 3.3.3 Influence of biological factors and capture method on protein expression

Principal component 1 for HPA axis proteins explained 88% of the variance. Model selection for HPA principal component 1(PC1) without the environmental variables suggested that HPA PC1 varied by region (F=4.8, p=0.004), capture year (F=3.4, p=0.07) and log total serum cortisol (ng/ml) (F=3.2, p=0.08) (Adjusted $R^2$=0.16, n=109). After controlling for total serum cortisol and capture year, bears from South highway 11 were found to have lower HPA PC1 compared to those originating from Swan Hills (p=0.003) (Figure 3.9). Model selection for HPA PC1 including the environmental variables suggested that HPA PC1 varied by region (F=6.0, p=0.005), log total serum cortisol (F=4.5, p=0.04) and anthropogenic change (F=5.0, p=0.03) (Adjusted $R^2$=0.21, n=49). After controlling for total serum cortisol and anthropogenic change, bears from North highway 16 were found to have elevated HPA PC1 compared to those originating from South highway 11 (p=0.004; data not shown due to low sample sizes).

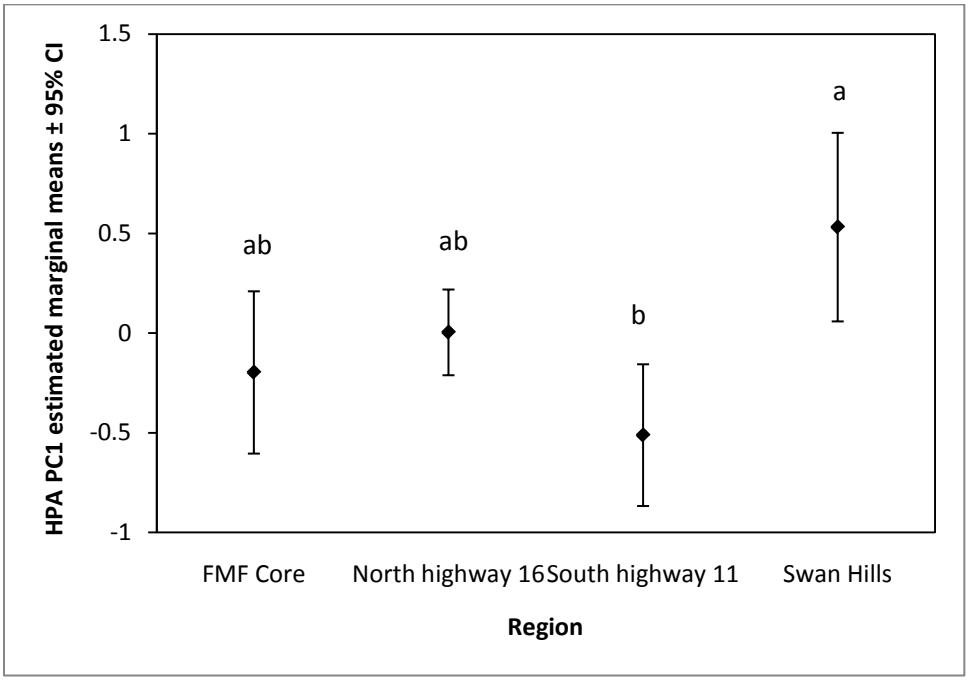


**Figure 3.9** Regional comparison of the first hypothalamic-pituitary-adrenal axis (HPA) principal component (PC1) modeled without environmental measures. Significant differences (p<0.1) are labeled with letters (a>b), as determined by repeated measures ANOVA followed by Sidak test. Abbreviation: CI, confidence interval.

Principal component 1 for ACC explained 83% of the variance. Model selection for ACC PC1 excluding the environmental variables suggested that ACC PC1 varied by region ($F=7.1$, $p<0.001$), log body length ($F=4.2$, $p=0.04$) and log total serum cortisol ($F=4.4$, $p=0.04$) (Adjusted $R^2=0.21$, $n=101$). After controlling for length and total serum cortisol, bears from Swan Hills were found to have elevated ACC PC1 compared to those originating FMF Core ($p=0.002$), North highway 16 ($p=0.02$) and South highway 11 ($p<0.001$) (Figure 3.10). Model selection for ACC PC1 including the environmental variables suggested that ACC PC1 varied by region ($F=6.7$, $p=0.003$), anthropogenic change ($F=5.5$, $p=0.02$) and road density ($F=3.1$, $p=0.08$) (Adjusted $R^2=0.24$, $n=52$). After controlling for anthropogenic change and road density, bears from North highway 16 were found to have elevated ACC PC1 compared to those originating from South highway 11 ($p=0.002$; data not shown due to low sample numbers).

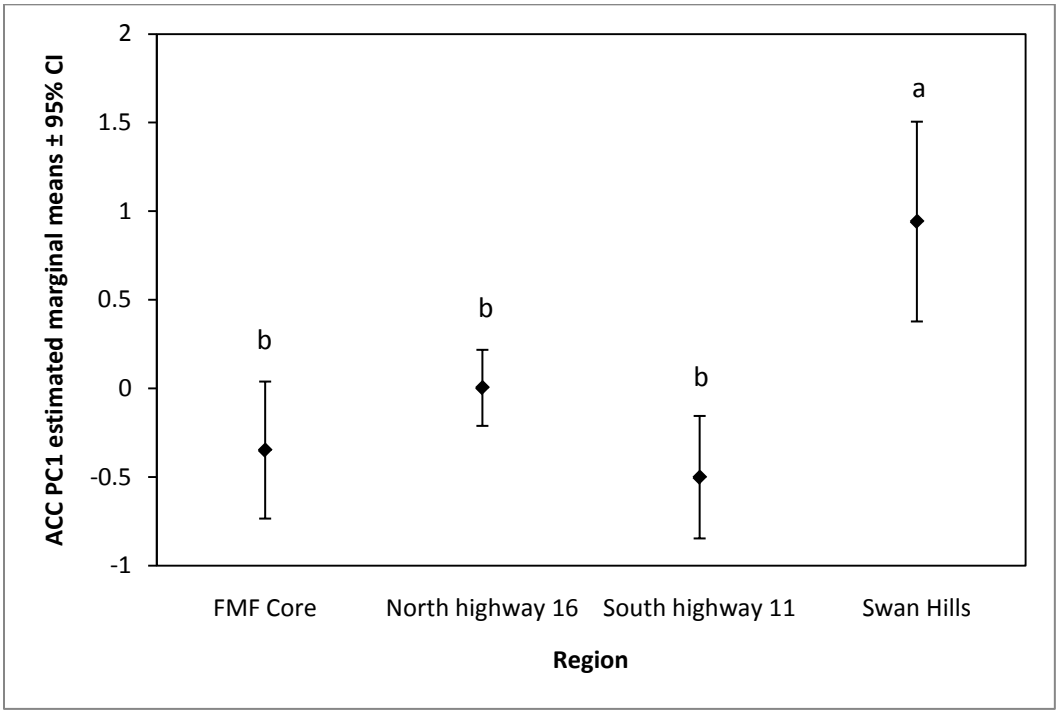


**Figure 3.10** Regional comparison of the first apoptosis and cell cycle (ACC) principal component (PC1) modeled without environmental measures. Significant differences ($p<0.1$) are labeled with letters (a>b), as determined by repeated measures ANOVA followed by Sidak test. Abbreviation: CI, confidence interval.

Principal component 1 for CS explained 48% of the variance. Model selection for CS PC1 excluding the environmental variables suggested that CS PC1 varied by region ($F=3.7$, $p=0.02$), log total serum cortisol ($F=5.0$, $p=0.03$), log mass ($F=5.0$, $p=0.03$) and log length ($F=10.6$, $p=0.002$) (Adjusted $R^2=0.21$, $n=89$). After controlling for total serum cortisol, mass and length, bears from Swan Hills were found to have elevated CS PC1 compared to those originating FMF Core ($p=0.01$) and North highway 16 ($p=0.02$) (Figure 3.11). Adding the environmental variables did not improve the CS principal component 1 model.

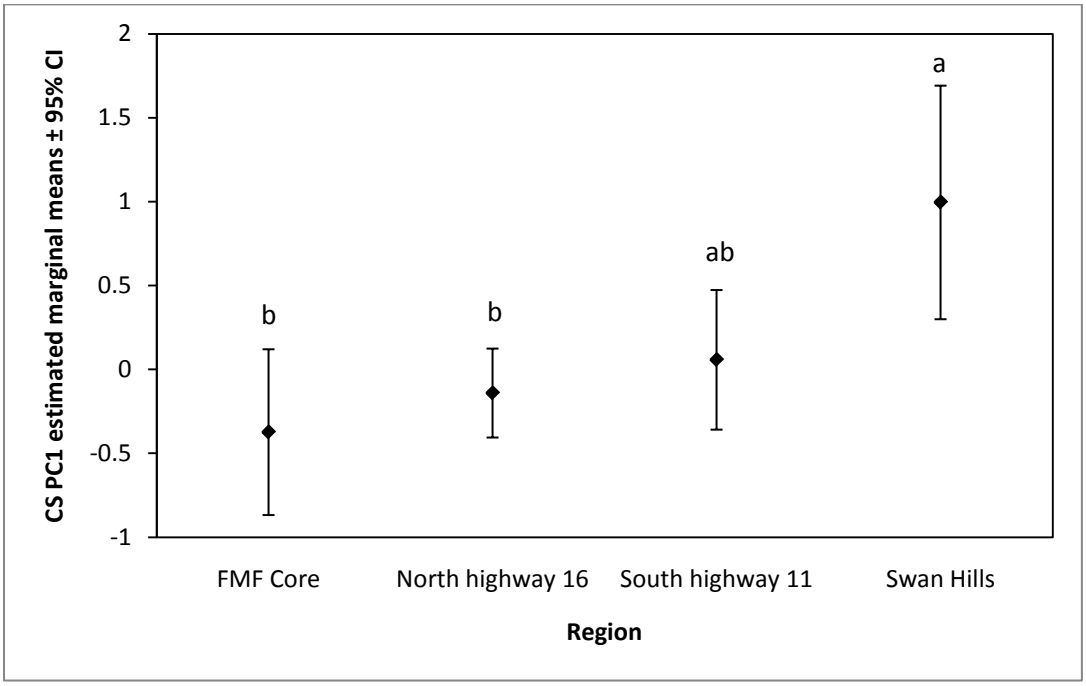


**Figure 3.11** Regional comparison of the first cellular stress (CS) principal component (PC1) modeled without environmental measures. Significant differences ($p<0.1$) are labeled with letters (a>b), as determined by repeated measures ANOVA followed by Sidak test. Abbreviation: CI, confidence interval.

Principal component 1 for OSI explained 69% of the variance. Model selection for OSI PC1 excluding the environmental variables suggested that OSI PC1 varied by region ($F=4.4$, $p=0.006$), capture year ($F=4.2$, $p=0.04$) and log total serum cortisol ($F=3.6$, $p=0.06$) (Adjusted $R^2=0.17$, $n=109$). After controlling for capture year and total serum cortisol, bears from Swan Hills were found to have elevated OSI PC1 compared to those originating FMF Core ($p=0.09$) and South highway 11 ($p=0.004$) (Figure 3.12). Model selection for OSI PC1 including the

environmental variables suggested that varied by region (F=5.0, p=0.01), log total serum cortisol (F=3.4, p=0.07) and anthropogenic change (F=6.6, p=0.01) (Adjusted $R^2$=0.19, n=49). After controlling for total serum cortisol and anthropogenic change, bears from North highway 16 were found to have elevated OSI PC1 compared to those originating from South highway 11 (p=0.009; data not shown due to low sample sizes).

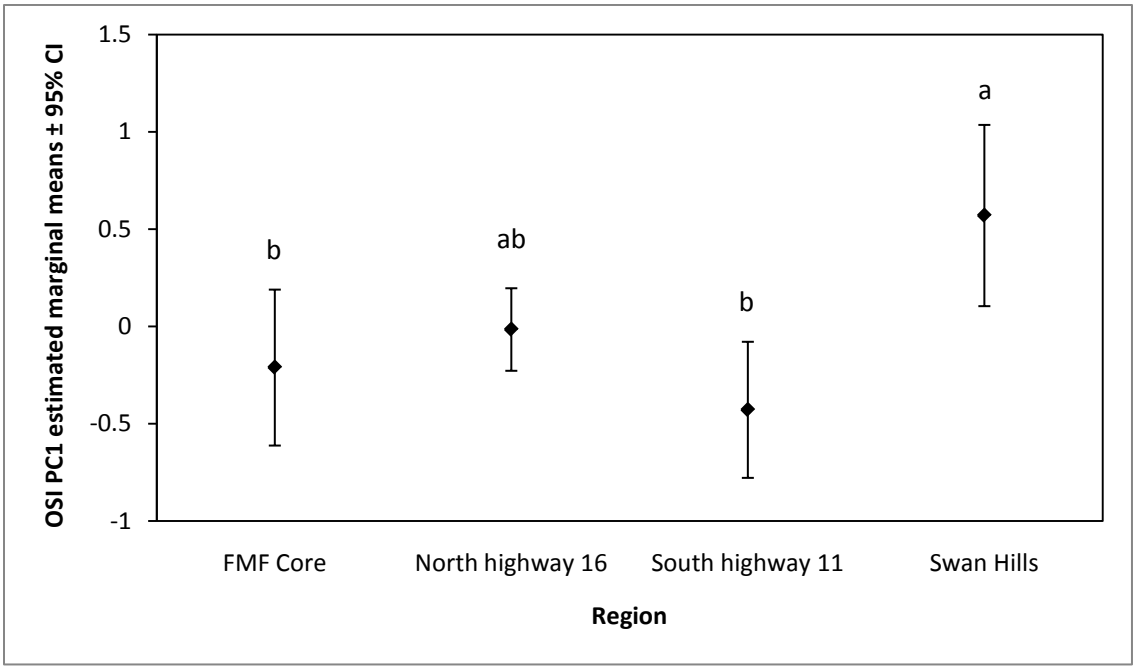


**Figure 3.12** Regional comparison of the first oxidative stress and inflammation (OSI) principal component (PC1) modeled without environmental measures. Significant differences (p<0.1) are labeled with letters (a>b), as determined by repeated measures ANOVA followed by Sidak test. Abbreviations: CI, confidence interval.

Model selection for stress protein index excluding the environmental variables suggested that the protein index varied by region (F=6.9, p<0.001), capture year (F=13.6, p<0.001) and log serum hsp60 (F=7.3, p=0.008) (Adjusted $R^2$=0.25, n=108). After controlling for capture year and serum hsp60, bears from South highway 11 were found to have depressed OSI index compared to those originating from North highway 16 (p=0.001) and Swan Hills (p=0.002) (Figure 3.13). Adding the environmental variables did not improve the protein index model.

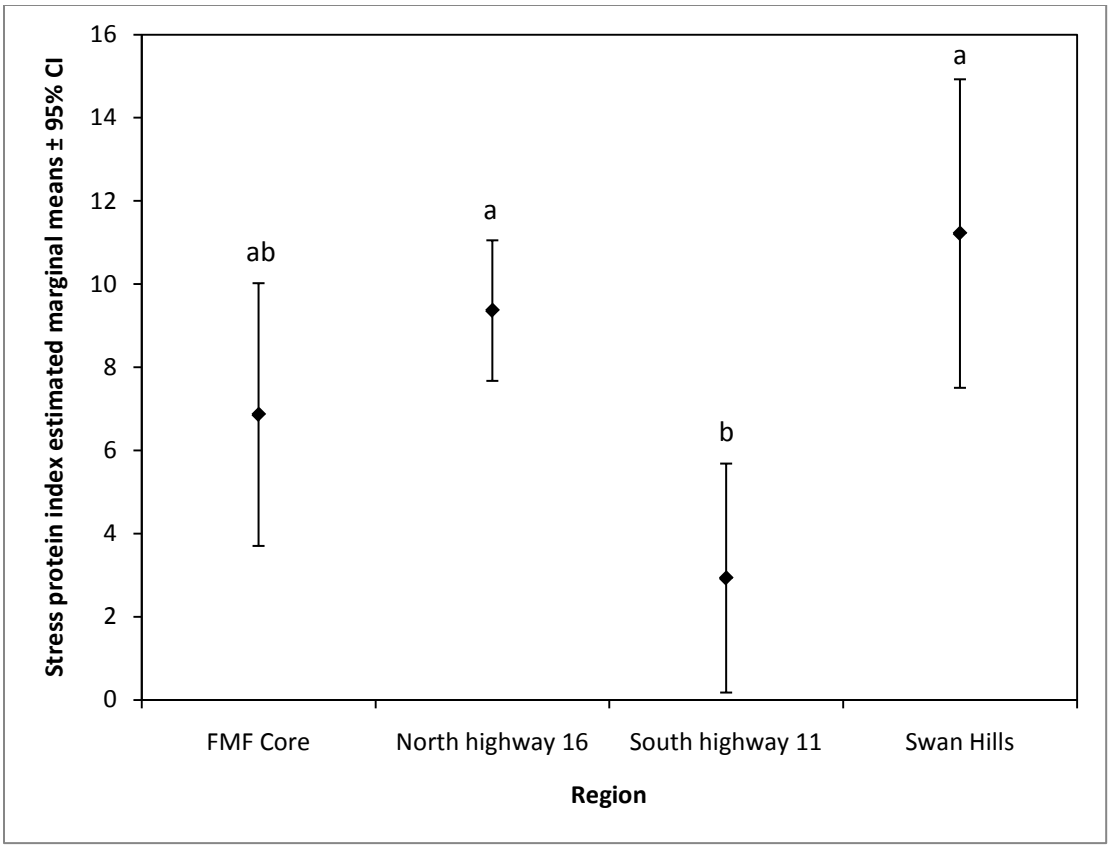


**Figure 3.13** Regional comparison of the stress protein index modeled without environmental measures. Significant differences ($p<0.1$) are labeled with letters (a>b), as determined by repeated measures ANOVA followed by Sidak test. Abbreviation: CI, confidence interval.

## Chapter 4 Discussion

In my research, an antibody-based microarray was developed to measure stress-associated proteins in grizzly bear skin. The microarrays were then used to evaluate stress protein expression in the skin of free ranging grizzly bears from Western Alberta. Relationships between stress protein expression in individual bears and other stress, health and environmental variables were then investigated using ANOVA modeling.

### 4.1 Relationships between stress proteins and environmental measures

Grizzly bears in Alberta consist of five genetically distinct groups, with divisions between populations coinciding with major traffic arteries, such as highways 1, 11 and 16 (Proctor and Paetkau 2004). Bears, especially females, have been found to be resistant to crossing highways (Proctor et al. 2005). The bears from FMF Core and South highway 11 are genetically independent groups while those from North highway 16 are somewhat isolated from Swan Hills, but immigration from North highway 16 to Swan Hills is suspected as evidence was found of some interchange of genetics between the groups (Proctor and Paetkau 2004, Boulanger 2009). These population units have home ranges that have differing degrees of anthropogenic influence (Alberta Sustainable Resource Development and Alberta Conservation Association 2010). Thus it was predicted that differential expression of stress proteins would be detected among population units. All principal component and stress protein index models for grizzly bear protein expression were able to detect regional differences in protein expression among bears from different population units in Alberta. This suggests proteomic changes due to differences in habitat and encountered stressors within the regions.

Historically, grizzly bears have not fared well as human activity expanded in their habitat along with resulting landscape modifications. Worldwide, grizzly bear populations have declined when their home ranges put them in contact with humans (Storer and Trevis 1955, Brown 1985, Naves et al. 2003). It was hypothesized that human-bear interactions would lead to stress and elevated stress protein profiles as well as increased mortality. Mortality and presumably human-bear interactions tend to increase in disturbed areas (Berland et al. 2008). A survey of British Columbia grizzly bears found those living on a plateau which was extensively modified by human activities had one quarter the density of those in nearby mountain habitats,

despite eating more high quality food items (Ciarniello et al. 2007). A similar finding was reported in Alberta with high densities of grizzly bears in the mountains and lower densities in the foothills which had been developed to a greater extent (Mowat et al. 2005). Two measures of human activity within the environment, anthropogenic change and road density, were found to be associated with the expression of stress proteins in grizzly bear skin in my thesis.

Anthropogenic alteration of habitat has been found to be detrimental to bear populations (Mowat et al. 2005, Ciarniello et al. 2007, Berland et al. 2008). Anthropogenic changes in a bear's environment were hypothesized to be a factor influencing stress protein expression in bear skin. Anthropogenic change was found to be a covariate for HPA PC1, ACC PC1 and OSI PC1 models. It was predicted that bears sampled from the South highway 11 region would have the highest protein expression measured when compared to the other regions in models that included anthropogenic change. The South highway 11 region is one of the most extensively developed landscapes in which grizzly bears still persist in Alberta, and grizzly bears from in and around Banff National Park have previously been reported to have the lowest reproduction rate of any studied grizzly population (Garshelis et al. 2005). However an inverse pattern to this prediction was observed, with expression of the protein category principal components and indexes being lower in the South highway 11 bears compared to bears originating in the North highway 16 region.

The hypothesized positive association between stress protein expression and anthropogenic change was not found. The relationship between HPA PC1, ACC PC1 and OSI PC1 and anthropogenic change was found to be negative. The relationship between bear habitat use and anthropogenic change is complicated as grizzly bear habitat preferences sometimes overlap with human development and activities, which increases the likelihood of human-bear interactions (Gibeau et al 2002). Wildfire creates open canopy areas in the forest that have been found to increase the production of food items that grizzly bears prefer, but historically wildfires have been suppressed in the Alberta foothills since the 1940s (Hamer and Herrero 1983, 1987, Raine and Riddell 1991, Hamer 1996a, 1996b, Nielsen et al. 2004c). Prescribed fires have been used in Banff National Park to simulate the landscape changes that would happen if wildfires were not suppressed, but at levels well below the historical average of natural burns (Green et al. 1996). Lower amounts of open canopy have resulted in a decrease of some major grizzly bear

food items, such as buffaloberry (Hammer 1996a, 1996b). Grizzly bear habitat quality may be increased by cutblocks resulting from forestry that stimulate the production of certain grizzly bear foods, but the roads created diminish the positive impacts (Nielsen et al. 2004c, Nielsen et al. 2008). Female grizzly bears from an area with extensive forestry activities were found to have better body condition than those from a relative undeveloped area (Ciarniello et al. 2009). Human activity through addition of cutblocks can increase the quantity of preferred bear foodstuffs but increases the risk of human-bear interactions. Forestry and associated cutblocks require roads, which have been found to be detrimental to bears (Boyce et al. 2009). Thus, the negative association between anthropogenic change and stress protein expression may have been strongly influenced by food availability.

Development of roads has been found to have indirect and direct negative effects on wildlife. There is worldwide concern about the impact of roads on wildlife (Spellerberg 1998, Fahrig and Rytwinski 2009). Certain species have been predicted to respond negatively to road develepment, such as species attracted to roads or showing no avoidance of roads, species with large home ranges, low reproductive rates and low natural densities and small animals that avoid habitat due to traffic disturbance (Fahrig and Rytwinski 2009). Roads have been found to negatively affect a variety of biota, including plants, insects, birds and mammals (Spellerberg 1998). Road development can lead to direct loss of habitat, fragmented habitat, the isolation of populations and mortality (Spellerberg 1998). As mentioned previously, highways have been found to limit bear movement (Proctor and Paetkau 2004, Alberta Grizzly Bear Recovery Team 2008). Roads into grizzly bear habitat have been found to be detrimental because of increases in human caused mortality risk (Nielsen et al. 2008, Alberta Grizzly Bear Recovery Team 2008, Boyce et al. 2009). Human access to grizzly bear range in Alberta has been increased as roads have been added to allow for natural resource extraction (coal, natural gas, oil and timber) and is projected to increase further in the next couple of decades (Nielsen et al. 2008). The majority of grizzly bear mortalities are related to human access and are human-caused (McLellan et al. 1999, Benn and Herrero 2002, Nielsen et al. 2004b). Benn and Herrero (2002) found that 90% of the grizzly bear mortality in their Alberta study was caused by humans, and the locations of death were within 500m of a road or 200m of a trail. It has been suggested that decommissioning roads to equal the number being added to areas previously unaccessed may maintain critical habitat for grizzly bears (Nielsen et al. 2006). Within the habitat of this study, the foothills of

Alberta, road construction is progressing at a rapid pace (Schneider 2002). It was hypothesized that road density would affect protein expression. The impact of road density was detected in the modeling of ACC PC1, with greater ACC protein expression associated with increasing road density. It was hypothesized that the bears originating from the Southern highway 11 region would have a higher expression in models incorporating road density due to the high amount of human development of the landscape described above. The opposite pattern was observed in bears from North highway 16, where they were found to have elevated ACC stress protein expression compared to South highway 11 bears. The predicted habitat deficit, highest food availability combined with displacement of bears from a habitat, was high for the lower foothills of North highway 11 and Swan Hills (Nielsen 2010). It would be worthwhile to see future modeling including the South Highway 11 area and see if it fit the pattern of expression found for the protein expression models that included anthropogenic change or road density.

Although road avoidance has been found to occur, grizzly bears have been found in several studies to use habitat closer to roads (Wasser et al. 2004, Roever et al. 2010). Roads that bears were more likely to be found were associated with habitats attractive to bears, such as cutblocks (Roever et al. 2010). Male grizzly bears living in the mountains were hypothesized to use primary and secondary logging roads for ease of travel as well as for the foraging opportunities presented by early-seral vegetation associated with roads, when the resource selection models found bears closer to the roads than random encounters would suggest (Ciarniello et al. 2007). It was suggested that the increased distance traveled between successive telemetry locations taken at 4 hour intervals, step length, observed could be a reaction to reduce the time spent by bears at risk near roads (Roever et al. 2010). Speed and traffic volume may affect how much a bear uses habitat near a road. It has been found that high speed, high volume highways detract from the attraction of bears to high quality habitat, possibly due to noise (Gibeau et al. 2002). Female grizzly bears were found further away from high volume traffic, but closer to human settlement, which brings its own risks of human encounters (Gibeau et al. 2002). It has been recommended that in Alberta open road density threshold be set at 0.6 km $km^{-2}$ for core grizzly bear habitats (Alberta Grizzly Bear Recovery Team 2008). Yet even in an area with road densities below the proposed 0.6 km $km^{-2}$ threshold, a smaller grizzly bear population than expected was observed, and it was suggested that perhaps attractants should be restricted more strictly as well (Roever et al. 2010).

The proportion of a bear's habitat that is protected was predicted to be negatively related to protein expression. This was not found to be the case as no model of principal component or index included proportion of habitat protected. If a bear's habitat encompasses both disturbed and undisturbed land, it could be at high risk since during certain foraging seasons, notably hypophagia (hibernation), grizzly bear use of disturbed areas increases (Berland et al. 2008). Disturbed areas, such as clearcuts and roadsides, can provide a diverse array of grizzly bear foods, such as tubers and roots, insects and plants (Nielsen et al. 2004c, Roever et al. 2008). Recreational activity on protected lands has been found to have negative impacts on wildlife (Hornocker and Hash 1981, Titus and VanDruff 1981, MacArthur et al. 1982, Copeland 1996, Hamann et al. 1999, Olliff et al. 1999, Arlettaz et al. 2007, Reed and Merenlender 2008). Recreational activity may have been one of the factors adding to the variability of stressors encountered by bears within the protected habitats.

Mean RSF is the probability that a bear will use a resource unit within its home range (Boyce 2006). A resource unit is a location in the landscape that an animal selects for use (McLoughlin et al. 2006). It can designate where the bear is, but not why (Boyce and McDonald 1999). Resource selection functions are statistical models that estimate the probability of use of a resource unit relative to the availability of that resource in the environment (Boyce 2006, McLoughlin et al. 2006). Patterns of habitat use have been observed to vary seasonally, which can result in separate RSF models for each season (Nielsen et al. 2004a, Boyce 2006). Mean RSF avoids the seasonality detected in RSF measurements by averaging 3 seasons. It has been suggested that RSF can describe the extent of habitat use and might hint at quality and abundance of resources in the area (Boyce and McDonald 1999). Increasing mean RSF indicates a greater probability of selective use of the environment versus random use of a bear's home range. There is concern that RSF models may not relate to population measures, such as density, or individual health and some have begun to question their utility for management of populations (Nielsen et al. 2005, Johnson and Seip 2008). No selectivity of habitat use was detected with principal component models or the stress protein index in my thesis.

Habitat alteration and human activity within the environment are factors reported to be stressors for wildlife (Creel et al. 2002, Homan et al. 2003, Arlettaz et al. 2007, Martinez-Mota et al. 2007). Human activity has also been found to negatively influence wildlife use of available

habitat (Johnson et al. 2005). Long-term stress has been found to decrease reproductive success in wildlife, which potentially impacts population health and can lead to a lag in detection of the problem (Chapman and Lambert 2000, Cyr and Romero 2007). Grizzly bears, with their large body size, low fecundity and large home ranges have a greater chance of population declines due to habitat alteration and other stressors (Cardillo et al. 2005). There is a time lapse, sometimes decades, between anthropogenic habitat alteration and declines in wildlife population health (Struhsaker 1976, Findlay and Bourdages 2000). For species of concern, such as the grizzly bears in Western Alberta, the custom protein microarray is a potential tool for rapidly assessing alterations in cellular stress which could then be extrapolated to individual health. Establishing linkages between cellular changes and population level processes is a huge challenge for conservation physiology (Cooke and O'Connor 2010). One fortunate element of being associated with such a large project studying a species from many different approaches is that modeling has been performed to determine if the estimated population densities observed are the densities that the habitat can support. Unfortunately this information, which is costly to obtain, may not available to all wildlife researchers (Chapman and Lambert 2000).

## 4.2 Relationship between stress proteins and health measures

### 4.2.1 Body measures

The observed relationship between lower body length and higher stress protein expression may suggest stress-induced inhibition of growth. Growth hormone secretion is inhibited by the signal cascade triggered by stress, and long-term stress can result in depressed growth (Wingfield and Romero 2001, Tsigos and Chrousos 2002). Body length was found through first principal component modeling to have a negative relationship with ACC and CS proteins. If the protein categories are reflecting chronic stress, then a negative association with growth would be predicted.

Body mass has been found to alter the response to stress. It has been suggested that animals with lower body masses and thus energy reserves may have a slower increase in glucocorticoid levels and a delayed recovery to baseline levels (Heath and Dufty 1998). Stress has also been found to alter body mass. Repeated stress exposure has been observed to induce temporary hypophagia and a long-term suppression of weight (Harris et al. 2002). Translocated

wild birds were reported to have reduced body weight and decreased sensitivity of the HPA axis to negative feedback (Dickens et al. 2009). Modeling of CS PC1 revealed a positive relationship with body mass. With hibernation losses and hyperphagic gains the body weight of a grizzly bear can vary a great deal during the year. Weight changes would be expected to vary in response to food available within the bear's home range and as mentioned above may vary with stress. Grizzly bear weight can fluctuate 20-70% seasonally, with the greatest weight change occuring in older females over the winter period due to the demands of gestation and lactation (Schwartz et al. 2003). The nutritional demands of bears are high, with a peak over summer and fall when individuals accumulate fat to survive winter (Rode et al. 2006, Berland et la. 2008). Female bears with young have an additional nutritional demand in the spring (Forley and Robbins 1995, Rode et al. 2001). Body length does not fluctuate as greatly, although rates of growth can vary due to age and yearly growth rate, which can be influenced by food availability and potentially stress. The positive association of CS PC1 with body mass may be related to higher food availability in a more stressful environment as serum cortisol was also found to have a positive relationship with CS PC1.

Body condition index is based on the standard residuals from body mass and body length, has been found to reflect body condition (fat and muscle mass), and is independent of body size (Cattet et al. 2002). It was not unexpected that the stress protein relationships to BCI were not identical to those of body length or mass. No optimized models for stress protein categories demonstrated a relationship to BCI. Heterogeneity within the bear populations may have masked any relationship with true body condition, or alternatively protein expression may be more influenced by factors that also affect length and mass on a shorter time scale.

### 4.2.2 Serum measures

One complicating factor when studying grizzly bears in the wild is the uncertainty of frequency of exposure and the bear's perceptions of potential stressors in the environment. The degree of HPA and sympathetic nervous system activation depends on the stress duration, intensity and type (Dronjak et al. 2004). Cortisol and ACTH expression are increased rapidly after the onset of stress; however, certain long-term stressors also result in elevated ACTH and cortisol release (Dronjak et al. 2004). Repeated exposure to a low to moderate stressor can lead to habituation with low to no HPA activation (Armario 2006). Repeated exposure to a severe

stressor can lead to a partial habituation of the HPA axis with the degree of habituation negatively related to the interval between exposures and intensity of stressor (Armario 2006). Certain stressors do not elicit habituation with repetition but rather produce an enhanced response (Armario 2006). Assessment of glucocorticoid levels has been one of the traditional assessments of whether a wildlife population is under threat from human activity (Arlettaz et al. 2007); however, a review of the literature found that the relationship between baseline glucocorticoids and fitness of individuals or populations is not consistent for all populations (Bonier et al. 2009). The expression of proteins as modeled by the first principal component found a positive relationship with total serum cortisol for all four stress protein categories. Increased protein expression associated with increased total serum cortisol could reflect acute, repeated or long-term stress; however the lack of association with capture stress combined with the association with landscape measures supports the notion that skin stress protein expression is reflecting longer term stress.

Acute psychological stress has been positively associated with serum hsp70 (Fleshner et al. 2004). Serum hsp70 has been found previously to differ in grizzly bears from different regions in Alberta and thus may also be able to reflect longer term stress (Cattet et al. 2008c). Controlling for capture method, it was found that serum hsp70 was inversely correlated to the proportion of the grizzly bear home range that was protected from human activity (Hamilton 2007). Bears whose home range was $\leq$15% protected from human activity were found to have elevated serum hsp70 (Hamilton 2007). Percent protected habitat is a complex variable, since protected habitats often have less food available seasonally due to wildfire suppression, edges of protected habitat may have greater human encounters, and increased mortality and recreational activity within protected habitats have been found to create stressful situations for wildlife. Stress protein functional groups and the protein index were not found to have a relationship to serum hsp70 levels or proportion of grizzly bear home range that was protected. The lack of relationship with serum hsp70 could reflect the lack of relationship found within the models for the proportion of habitat within a grizzly bear's home range that was protected.

Serum hsp60 has been reported to increase with a variety of longer term stressors such as cardiovascular disease, artificial enlargement of bird brood size, psychological stress in humans, and was inversely associated with psychosocial measures such as socioeconomic status (income),

indicating a responsiveness to chronic stress (Lewthwaite et al. 2002, Pockley 2003, Merino et al. 2006, Shamaei-Tousi et al. 2007). Another study of Alberta grizzly bears found that serum hsp60 did not reflect regional location of the bears (Cattet et al. 2008c). In contrast, the stress protein index was found to have a positive association with serum hsp60 and included a regional component. Differing hsp60 and hsp70 associated changes are not surprising as hsp60 and hsp70 have distinct actions within the body (Henderson 2010). Extracellular hsp60 and hsp70 may be signals of cellular trauma and stress to any particular part of an organism as well as immunomodulators, but what stresses they respond to and their actions within the body may differ (Calderwood et al. 2007, Pockley et al. 2008, Henderson 2010).

Serum GGT was not found to have a relationship with skin stress protein expression. Serum GGT is often used in human medicine as a marker of liver dysfunction, and has also been associated with cardiovascular risk, diabetes, kidney disease and cancer (Targher et al. 2010). It has been suggested previously that serum GGT may be a marker for oxidative stress (Lee et al. 2004). The changes in serum GGT associated with oxidative stress in human studies were within the normal physiological range (Lee et al. 2004). Serum GGT in wildlife has generally not been found to vary with the stressors of parasites, reproduction, handling, capture or captivity (St. Aubin et al. 1979, Weber et al. 2002, Barnes et al. 2010, dos Santos Schmidt et al. 2010, Topal et al. 2010). However there are other studies examining different species and capture methods that do show serum GGT differences with capture type and positive correlations with time between capture and sampling, although within the normal reference range of the species (Kreeger et al. 1990, Omsjoe et al. 2009). The inherent variability of wildlife may have masked any relationship that may have existed between the OSI proteins and serum GGT as in human studies the GGT elevations were within normal physiological ranges.

### 4.3 Summary measures of protein expression

An HPA response alone cannot be interpreted as a stress response, as the HPA axis is involved in the homeostatic response to a stimulus (Armario 2006). The acute stress response is also vital to survival of wildlife, while chronic stress can result in pathology (Dickens et al. 2010). Stimulation of the HPA axis is not only involved in short-term adaptation to maintain homeostasis in the face of challenges, but is also involved in the normal day-to-day activities associated with increased locomotion, exploratory behaviour, appetite and food seeking

behaviour (McEwen et al. 1998, Sapolsky 1992). The altered expression of proteins involved in all four of the stress protein categories evaluated in association with various health and environmental variables gives a stronger indication that bears were encountering stressors in their environment, and the possibility of that such stress was causing dysregulation within the body. The challenge with interpreting proteomic changes within animals in the wild is the uncertainty of the stressors encountered and their potential physiological effects.

Allostasis is the body's attempt to maintain stability through the activation of physiological processes. Chronic elevated activity or inactivity of the physiological systems involved in allostasis can lead to wear and tear on the body and brain, which has been termed allostatic load (McEwen 1998). In human medicine, it has been found that an allostatic load index that incorporates a variety of physiological parameters was positively related to adverse health outcomes (Seeman et al. 1997, McEwen 2000, Seeman et al. 2004). Calculations of allostatic load have been based on distribution of each biomarker rather than clinical thresholds as many of the variables do not have accepted clinical cutoffs and the focus is on sub-clinical dysregulations (Seplaki et al. 2005). Human health was found to be more accurately reflected by allostatic load rather than the individual parameters used to calculate the index (Seeman et al. 1997, Seeman et al. 2004).

In this study, grizzly bear summary measures were calculated based on the same principles used to calculate allostatic load in humans. Different modeling techniques for understanding grizzly bear protein expression were compared. The more classical elevation and depression allostasis model was attempted with the top 10% and bottom 10% of expression incorporated with little success, as there were no regional effects found except for the CS functional group model when environmental measures were included and the models had a much lower fit to the data (Appendix 2, Adjusted $R^2$ = 0.04-0.18). A variant of the allostatic load approach found was also attempted based on a recent publication. Summation of the extreme scores at one tail of the biomarker distributions was found to perform better than summation of extreme scores at both tails (Hampson et al. 2009). This agrees with my findings, as the model that only accounted for the top 25% of protein expression had a higher fit measure (Appendix 3, Adjusted $R^2$ = 0.15-0.28), detected regional effects, and had similar findings to the first principal component models. The stress protein index was calculated from the expression of all 31

proteins on the array, which encompassed physiological processes such as apoptosis, cell cycle, cellular stress, oxidative stress, inflammation and the HPA axis. The stress protein index was chosen as the summary measure for all of the functional protein categories.

The model chosen for each stress protein functional category was the first principal component determined by principal component analysis. Principal component analysis is used to condense information from a large number of original variables (McGarigal et al. 2000). The expression of 31 stress proteins in skin samples from over 100 bears represents a large data set, and presents challenges in interpretation. Condensed measures are easier to present and interpret when attempting to investigate relationships between protein expression patterns and measures of grizzly bear health and environment. Multivariate techniques are problematic due to dependencies among variables, and principal component analysis can be used to handle the dependencies by creating new independent variables (McGarigal et al. 2000). The first principal component was chosen for the model as it explains the maximum amount of variation possible in one dimension (McGarigal et al. 2000). The first principal components were able to account for 88, 83, 69 and 48 percent of the variation for the HPA, ACC, OSI and CS proteins, respectively. Both principal component analysis and grouping by functional categories has been performed previously for DNA microarray data (Mootha et al. 2003, Pavlidis et al. 2004, Subramanian et al. 2005, Kong et al. 2006). Grouping of genes, using a priori knowledge, facilitates interpretation of results such that the effects seen without grouping may miss weak effects (Mootha et al. 2003, Kong et al. 2006).

In agreement with the results of my thesis were the findings of Southern et al. (2002), who reported elevated expression of OSI (COX2, iNOS, SOD1, SOD2), HPA (GR), ACC (caspase 8), and CS (grp75, hsp25, hsp40, hsp90) proteins in the skin of spotted dolphins (*Stenella attenuate*) stressed by fisheries. Dolphins stressed by fisheries have also been found to have physiological damage in the form of focal heart lesions (Forney et al. 2002). Cardiac health has been found to be altered by chronic stress (Dickens et al. 2010).

Fitting the current study into the reactive scope model, which has been proposed to be a next step in the evolution of stress modeling, after allostasis, is more complex (Romero et al. 2009). The reactive scope model calls for a young, naïve animal to provide the limit to the reactive homeostasis for that mediator to determine at what level homeostatic overload begins

(Romero et al. 2009). How does one fit non-domesticated organisms into the model? One of the problems encountered with this study was the challenge of working without a classic control. How does one find a grizzly bear that is not stressed? Zoo animals were considered, but the significant psychological stress in animals confined in zoos, especially large mammals (Terio et al. 2004, Clubb and Mason 2003, Mason and Veasey 2010), raised concerns over this approach. Even if zoo animals were used as a control, one would have to find a number of zoos willing to allow application of light to moderate stressors and skin sampling. Using a closely related animal as an alternative could also lead to confusion if life histories were not taken into account. For example, two species within the family Sciuridae, red squirrels (*Tamiasciurus hudsonicus*) and Arctic ground squirrel (*Spermophilus parryii*), with differing life histories were found to have differing stress responses (Boonstra and McColl 2000).

Allostatic load calculations have been employed in the human health field and were found to account for more variance than the separate parameters evaluated, which was thought to reflect the allostatic load score encompassing information from multiple biological pathways (Seeman et al. 2004). Human health studies have reported allostatic load to reflect increased risk of cardiovascular disease, poor cognition and physiological functioning (Seeman et al. 1997, Evans and Schamberg 2009, de Castro et al. 2010, Mattei et al. 2010). Allostatic load scores have been observed to reflect chronic stress that people experience, such as poverty (Evans and Schamberg 2009, Bird et al. 2009). Due to concern about whether human influence on natural ecosystems is causing conservation issues, there is a call for assessment of stress in wildlife (Wikelski and Cooke 2006). Evidence of increased allostatic load has been found in other mammals. A study of dominance hierarchies in male cynomolgus monkeys (*Macaca fascicularis*) found an elevation of blood pressure and accelerated atherosclerotic plaque formation in males vying for position associated with the release of catecholamines (McEwen and Seeman 1999). There has been a call in the literature to evaluate the effects of allostasis at the cellular level, such as changes in protein expression (McEwen and Seeman 1999), and my thesis research has contributed to this emerging area by investigating changes in stress protein expression in free-ranging grizzly bears.

### 4.4 Two dimensional gel electrophoresis

Alteration of mitochondrial protein expression, which has been linked to chronic stress, was discovered in grizzly bear skin and muscle using two dimensional gel electrophoresis. Several of the proteins found to be altered between grizzly bears under stressful conditions compared to bears theorized to be less stressed, such as components of the ATP synthase complex, are related to mitochondrial function. Chronic stress has been found to cause mitochondrial dysfunction as well as alteration of mitochondrial protein expression (Duclos et al. 2001, Madrigal et al. 2001, Liu et al. 2004, Manoli et al. 2007). These mitochondrial proteins altered by stress are worth further investigation as mitochondria seem to be affected by many diverse stressors in fish and mammals (Moens et al. 2006, Mancia et al. 2008, Galindo et al. 2009). To date no antibodies have been found that react to ATP synthase proteins in grizzly bear tissue, but validation testing will continue. Future refinement of antibodies on the grizzly bear microarray I developed should add antibodies related to mitochondrial function. The two dimensional gel electrophoresis resulted in one antibody added to the array, however it did hint to a functional group of proteins involved in mitochondria activity that would be an interesting addition to future versions of this microarray. The inability to find an antibody to evaluate mitochondrial activity highlights the difficulty of finding commercial antibodies that can be validated for grizzly bears.

### 4.5 Commercial vs. custom protein microarrays

Custom protein arrays may be the most feasible way to monitor stress protein expression in wildlife. The commercial antibody arrays were found to have low reactivity and many of the antibodies from the commercial arrays, when validated by western blotting, did not react to the target antigen in grizzly bear tissue. A large number of antibodies (253) that were screened and rejected during the developmental phase of the microarray was also an indication that use of untested antibodies could lead to erroneous protein expression data. Extreme caution should be employed when using commercial antibody arrays designed for use with laboratory animals or humans for wildlife without validation of the antibodies on the chip. Concern about cross-reactivity and divergent sequences resulting in failed hybridization has led to a call for species-specific DNA microarrays as well (Kennerly et al. 2008).

Custom protein and DNA microarray work has been used to assess stress in other wildlife species. A DNA microarray study of wild bottlenose dolphins (*Tursiops truncatus*) acutely

stressed by capture-release found increased expression of energy metabolism, stress and trauma responsive genes, as well as down regulation of certain immune related genes (Mancia et al. 2008). Protein profiling using a reverse array, in which the protein is fixed to slides and flooded with an antibody cocktail, was able to differentiate between diseased and healthy African elephants (*Loxodonta africana*) (Bechert and Southern 2002). Protein profiling, also using a reverse array, was able to differentiate between spotted dolphins stressed by fisheries and those unaffected (Southern et al. 2002). European wild boar (*Sus scrofa*) were found to have gene expression affected in functional categories including intermediary metabolism, apoptosis, immune response, cell growth and protein synthesis when infected by *Mycobacterium bovis* (Galindo et al. 2009). Endocrine disrupting compounds have been found via DNA microarray to alter the expression of many genes in common carp (*Cyprinus carpio*) including hsp60, cytoskeletal genes and those involved in mitochondria function (Moens et al. 2006). Rainbow trout (*Oncorhynchus mykiss*) exposed to various stressors were reported to have altered liver gene expression (Momoda et al. 2007). Custom protein and DNA microarray work conducted in a variety of species have demonstrated the utility of these tools when assessing the impact of disease, chemical insult and anthropogenic stress. Protein arrays avoid one of the major criticisms of DNA microarrays, with respect to the large differences detected between the transitory message, mRNA, and the effectors in the cell, proteins (Wastling et al. 2009, Lundberg et al. 2010).

### 4.6 Tissue comparison

Skin was selected as the optimal tissue for analysis. This was due to the collection of skin from the ear in conjunction with the placement of ear tags, the less invasive procedure of obtaining the tissue compared to muscle collection, and for its ability to be remotely sampled. The use of remote biopsy darts to collect skin samples is an exciting possibility for the future of wildlife microarray use. The darts collect small samples remotely without the need for anesthesia or the stress of capture. Dr. Marc Cattet has begun investigation of use of these darts on grizzly bears with the collection of dart samples from several of the snared grizzly bears (data unpublished). Biopsy darts have been successfully used for skin biopsy collection from cetaceans, elephants, hippopotamus (*Hippopotamus amphibious*) and South Andean deer

(*Hippocamelus bisulcus*) (Roca et al. 2001, Beckwitt et al. 2002, Bechert and Southern 2002, Dizon et al. 2002, Tovar et al. 2008).

Skin has been found to have a stress response cascade that mirrors the HPA axis (Slominski et al. 2000, Arck et al. 2006). It has been proposed that the skin, nervous, endocrine and immune systems are a large multidirectional complex rather than autonomous units (Reich et al. 2010). The stress protein expression profiles of grizzly bears in my thesis research were consistently higher for the skin compared to muscle. Skin sampling location was not found to affect the expression of array proteins. Contrasting with this finding, hair cortisol concentrations in grizzly bears have been found to vary across different body locations, with neck hair having higher concentrations (Macbeth et al. 2010). Skin consists of a variety of cell types, some of which form the peripheral mimic to the central HPA axis. Fibroblasts, melanocytes and hair follicles produce cortisol (Ito et al. 2005, Slominski et al. 2007). The skin samples used for this study had the hair shorn off at the skin level. The skin cells sampled would have consisted of a variety of cells including the keratinocytes, melanocytes, Langerhans cells, Merkel cells, hair follicles and fibroblasts (Boulais and Misery 2008). Perhaps the heterogeneity of cells in skin samples resulted in the lack of difference seen between skin sampling locations, contrasting with the hair cortisol concentrations resulting from the hair follicle contributions. Future analysis of skin protein expression and hair cortisol expression patterns in relation to environmental measures is recommended to determine if the patterns are correlated. Based on this information, it appears that skin biopsy samples can be collected from a variety of body locations without influencing results. Greater expression of stress proteins from skin compared to the muscle is ideal for wildlife research as there is concern for wildlife welfare which results in researchers and wildlife managers wanting to create the smallest wound possible to minimize infection risk and speed healing. The skin sampling procedure was much less invasive than the procedure for collecting muscle, and skin is currently collected in many wildlife studies currently as ear tags are inserted to aid animal identification.

A link between central HPA activation and effects on the skin has been detected in humans. Psychological stress has been associated with comprised skin barrier function, decreased epidermal proliferation and decreased expression of various proteins (involucrin, loricrin and filaggrin) leading to thinner epidermis (Slominski et al. 2008). Psychological stress

has also been linked to the severity of psoriasis in humans. Interestingly, patients with greater stress levels had lower serum cortisol compared to the patients reporting less stress, yet still had stress effects on the skin (Evers et al. 2010). In addition to the skin stress axis reacting to HPA activation, stress can cause an influx of immune cells to the skin (Reich et al. 2010). Psoriasis is an autoimmune condition of the skin. Activation of the autonomic nervous system and psoriasis has been correlated (Chapman and Moynihan 2009).

The skin, endocrine, nervous and immune systems are revealing themselves to be a large complex system with multidirectional communication (Reich et al. 2010). Physiological systems (e.g. immune, metabolic and endocrine) react and influence each other in relation to selection pressures in the environment, such as disease, food availability, predation and environmental unpredictability (Ricklefs and Wikelski 2002). Bidirectional communication occurs between the central nervous system and the immune system (Glaser and Kiecolt-Glaser 2005, Chapman and Moynihan 2009). Acute and chronic stress, anxiety, and depression have been linked to changes in innate and adaptive immune responses (Glaser and Kiecolt-Glaser 2005). Psychoneuroimmunology is the study the complex interactions of the central nervous system, endocrine and immune systems (Glaser and Kiecolt-Glaser 2005). Stress-related immune dysregulation results in delayed wound healing, and altered pathophysiology of viral infection, hypothesized to cause premature aging of immune cells (Glaser and Kiecolt-Glaser 2005). Evaluating skin protein expression could therefore be a very useful approach for investigating the complex interactions among different physiological systems in wildlife.

### 4.7 Protein quantity

Small skin samples (50 to 200 mg) contain sufficient protein to evaluate protein expression in grizzly bears. Ear plug samples, biopsy punch and biopsy dart samples all had sufficient protein quantities to label the tissue and run 80 μg in duplicate or triplicate wells of one microarray. The lowest protein concentration attempted in this study, 10 μg of sample protein and 10 μg of pooled standard in 230 μl volume, produced a detectable signal on the custom protein microarray, which is within the published limits of the sensitivity of fluorescent labeling of proteins captured by protein microarray, as proteins have been previously detected to a limit of approximately 1pg/μl (MacBeath 2002). The higher the protein amount incubated on the microarray, from 10 μg to 80 μg, the higher the relative protein expression measured with the

noted exception of 20 µg, which was found to produce lower measured protein expression for HPA, ACC and OSI proteins. Contributing to this effect may have been higher background fluorescence occurring in the 20µg samples without an increase in protein signal measured. Higher background fluorescence may have contributed to the larger variation in the 80 µg protein samples. Background fluorescence has been observed to be problematic for protein microarrays (Haab et al. 2001). Rolling circle amplification could be used in future iterations of this array to amplify the protein signal (Spisak and Guttman 2009). Two-color, rolling circle amplification procedures, which labels samples with small markers such as biotin or digoxigenin, would be preferential over the procedure requiring two specific antibodies for each protein (Zhou et al. 2004, Schweitzer et al. 2002). Validation of commercial antibodies to non-domestic species, such as wildlife, can have a low rate of validation requiring a great investment of time and money. Further work reducing the background fluorescence or adopting a method of amplifying the spot signal in the future is recommended.

### 4.8 Protein degradation

One concern with all protein work is the time from sampling to the time of freezing in order to preserve proteins from degradation. Significant changes in protein expression were noted for HPA and ACC proteins by 24 hours at room temperature for unpreserved grizzly bear skin. For the CS proteins an increase from 24 to 48 hours was observed. Protein degradation is not homologous, as it is influenced by parameters such as size, structure, composition and post-translational modifications (Spisak and Guttman 2009). Postmortem increases in protein expression of myosin light chain 1 and lactoylglutathione lysase along with decreases in hsp27 and hsp20 have been detected in bovine muscle by 2D electrophoresis followed by MALDI-TOF/TOF MS (Jia et al. 2006). In contrast another study of bovine muscle found an increase in hsp27 and decrease in hsp70 within 48 hours postmortem (Bjarnadottir et al. 2010). Generally, partially degraded protein molecules can bind to microarrays, but they usually have a weaker signal (Spisak and Guttman 2009). It is possible that degradation could have resulted in a change of solubility of the protein, which has been observed previously, or perhaps the protein in its native state was still partially in complex with other proteins as it is the cell and degradation released the protein (Perdew 1988, Rogalla et al. 1999, Bjarnadottir et al. 2010).

Preservation of samples collected in the field can be a challenge. In a laboratory setting samples are taken and frozen immediately, often at -80$^{o}$C. Even in the medical field, protein instability is a concern for protein microarray work (Spisak and Guttman 2009). Field work poses unique challenges to research monitoring protein expression, as the time between sampling and freezing is often prolonged. It is often hard to find space to bring ice, dry ice or liquid nitrogen on a helicopter, or carry enough storage materials to keep the samples cold or frozen when out in the field for a full day. Freezers at base camps are a luxury that was available for this project, but may not be available to all wildlife researchers. Grizzly bear population size in Alberta is low compared to a nearby population in Montana and a great deal of effort is expended capturing bears (Nielsen et al. 2009). Low population density makes use of opportunistic samples such as recently deceased bear carcasses of bears killed by cars and trains desirable. Three of the four protein categories demonstrated altered measurement of protein expression within one to two days at room temperature. This indicates that use of samples left for a day or more at room temperature, such as road kill or other incidental samples from dead animals may lead to increased variation in the microarray data leading to complications in interpretation.

Use of a preservative such as RNA-later may be an important addition to using protein microarrays to study stress in wildlife. The effects of degradation on measured protein expression may have been influenced by the prolonged exposure at ambient temperature that the samples available for this experiment underwent before freezing, so there may already have been protein degradation that occurred in these tissues. Investigation of preservatives such as RNA-later may make protein microarrays more practical for wildlife research. Preservatives would allow for small samples to be taken in the field without need for immediate refrigeration or freezing. No differences were detected after 24 hours for samples immersed in RNA-later compared to those that were not preserved although there was a trend for elevated protein expression with the preserved samples. Caution should be employed because of the significant finding of elevated measured expression with immersion in RNA-later at zero hours for HPA proteins and the trend for increased protein expression of ACC proteins. It is recommended that further testing should be done to determine if RNA-later preserves the proteins of interest and whether protein expression measured is artificially elevated by immersion in RNA-later. Another evaluation of RNA-later with samples that had not already undergone some time at

ambient temperature post-mortem may reveal a difference degradation with RNA-later preserved samples. RNA-later has been noted for its preservation of proteins in previous studies (Rodrigo et al. 2002, Barclay et al. 2008). RNA-later, and potentially other preservatives, may expand the ability of wildlife researchers to utilize technology evaluating protein expression, such as the microarray developed during my research.

### 4.9 Factors affecting protein expression

The grizzly protein microarray detected no differences in stress protein expression by sex, age or capture type. This corresponds with the findings of no effect of age, sex or reproductive class effects on serum hsp60 and hsp70 or total serum cortisol in grizzly bears (Hamilton 2007, Lindsjo 2009). Differing from the findings of this study, capture type has been associated with increased serum hsp70 and total cortisol levels in grizzly bears (Hamilton 2007, Lindsjo 2009). A lack of sex and age class variation was also found with manatees (*Trichechus manatus latirostris*) with serum cortisol highest in injured or diseased animals (Tripp et al. 2010). The lack of responsiveness of skin protein expression to capture (an acute stressor) could reflect a lag in skin stress axis responsiveness compared to the central HPA axis, or the skin stress axis responding more to chronic HPA activation rather than acute activation. Fecal glucocorticoid levels, which reflect glucocorticoid secretion in grizzly bears over time rather than acute pulsatile secretion into the serum which varies diurnally, have not been found to detect sex differences (Monfort et al. 1993, von der Ohe 2004, Wasser et al. 2004).

Unfortunately, because of terrain differences capture methods were not used evenly in each region. Within Swan Hills 100% of the bears were captured by snare, North Highway 16 66.1% of bears were captured by snare, South Highway 11 27.5% were captured by snare and FMF Core 15.8% were captured by snare. Total cortisol in serum has been found previously in grizzly bears to be affected by capture stress (Cattet et al. 2003, Hamilton 2007). Snaring bears has been found to result in indications of greater muscle injury and stress in bears indicated by higher serum cortisol levels and a stress-associated change in leukocytes compared to those darted from a helicopter (Cattet et al. 2003, Hamilton 2007, Cattet et al. 2008b). The observation that microarray proteins were not affected by capture type lends support that the array is not detecting changes in protein expression resulting from acute capture stress.

### 4.10 Antibody dilution, dye flipping and array variation

The information generated during the development of this protein array, such as decreasing measured protein expression and printing efficiency with increasing printing buffer concentration will contribute to the optimization of future custom wildlife arrays. The intra- and inter- assay variation were within acceptable criteria. The printing inconsistencies were likely to have contributed to the variation found for certain antibody features. It will be a priority to improve the printing consistency and lower the inter-assay variation for future generations of this array. The relative protein expression (sample fluorescence/pooled standard fluorescence) measured on the grizzly bear protein array has been found with other protein microarray studies to be unaffected by changes in spot size or density (Haab 2001). This may have helped counter any minor issues with printing efficiency. The flipping of the dyes resulted in differing relative protein expression. The fluorescent dyes Cy3 and Cy5 are known to have differing fluorescence characteristics (Mujumdar et al. 1993, Berlier et al. 2003). To avoid this variability, the dye choice should therefore be kept constant for the sample and reference pool.

### 4.11 Conclusion and future directions

Wildlife research is an exciting field at present with a wide selection of new techniques available and even more being adapted from the human health field. However, work with reclusive species with large territories may limit the techniques applicable. For instance, an immunoaffinity chromatography method has been developed to simultaneously evaluate a variety of neural and immune biomarkers in human sweat, collected through cutaneous sweat patches (Marques et al. 2010). This method is noninvasive, however cutaneous sweat patches have to be applied which would require the stress of capture and in order to retrieve the patches for analysis. The animal would have to be recaptured or the patches would have to drop off on their own, and retrieving the patches in a species that can travel for kilometers in a day would be arduous. A danger with using new technologies is over reliance on one technique to the exclusion of other information, which can result in a “technology trap”: quick results with limited biological information or interpretability (Nielsen et al 2010). The protein array is not meant to replace all other techniques for studying wildlife. The array would be a valuable addition to a wildlife researcher’s arsenal for evaluating whether a population is in need of further study. Protein expression in skin can be related to both anthropogenic environmental

change and health measures in grizzly bears. Another area the array may be useful is to evaluate whether there is an improvement after ecological restoration activities. There has been a call for integration of physiology measurement and ecological restoration within the literature (Cooke and Cory 2008). Use of physiological stress measures has been limited on free-ranging animals due to the invasiveness and potential for bias of capturing and withdrawing blood from wild animals (von der Ohe 2004). Skin samples taken remotely by dart would eliminate these concerns, and represents an important area for future research.

Time and funding did not allow for a lab based assessment of the custom array developed in my thesis. Future studies involving an unpredictable schedule of repeated stressors in a laboratory or field research station using a species with a more limited home range would further elucidate the changes in skin protein expression relative to long-term stress. It would be interesting to evaluate protein expression in the skin in comparison with other indicators of stress such as behavioural changes, adipose deposition and atherosclerotic plaque formation. A caveat should be recognized if one tries to use domesticated animals, as the human selection over generations for domestic animals to be calm and less prone to flight has attenuated their reactions to stress compared to their wild relations (Kunzl and Sachser 1999). With the great interest in assessing the health of genetically heterogeneous wildlife populations, perhaps a study could be conducted with logistically less challenging wildlife such as rodents or birds. Simultaneous evaluation of interacting systems, such as HPA axis, cellular stress, apoptosis, the cell cycle, oxidative stress and inflammation with noninvasive techniques may help elucidate the underlying reciprocal actions of these systems and their role in the progression of stress-related disorders.

In summary, the protein microarray developed in my thesis was able to detect regional differences in protein expression in small nonlethal skin samples, and the use of a preservative would make skin sampling more viable in the field. A “snap shot” of skin protein expression altered by long-term stress in individual grizzly bears represents a valuable tool for detecting potential population problems. This microarray has potential to be used for other wildlife species, as the antibodies were developed to target antigens from humans, rats, mice or cattle and were validated for grizzly bear proteins. The antibodies on the microarray would be predicted to

bind to a region of the protein that does not vary between widely divergent species, and would be expected to bind to the correct antigen in other wildlife species.

Future laboratory refinement, such as improvements in the printing of the arrays, could lead to lower array variation and expanded use of the array in the field. Wildlife research involves large genetic as well as habitat variation that are not encountered in laboratory studies. Refinement of the microarray would help balance the variability inherent with bears and their habitats. Further optimization of printing should be done to minimize the printing inconsistencies seen in this first trial of the custom grizzly bear microarray. A different printing buffer or additional purification of the antibodies prior to printing may help the consistency of the spot morphology. The RNA-later study should be repeated with a larger number of samples and with tissues frozen immediately after collection. The large grizzly bear skin samples available for initial array development and laboratory validation in this study were invaluable, but there was a lag time between the mortality of the bear and tissue sampling allowing time for protein degradation to possibly occur. With validation of the antibodies via western blotting for other species of mammals, perhaps the preservative testing could be repeated with an animal whose tissues were easier to obtain such as rats or cattle. Studying stress in a species such as the grizzly bear is an exciting challenge. The habitat of grizzly bears is in flux and as studies are conducted to mitigate the impacts of anthropogenic habitat change, the allostatic load of the bears may change. It is difficult to determine what grizzlies encounter as their habitat is sometimes densely forested, the bears have cryptic coloration and their territories are vast. Therefore, work has commenced involving video cameras on bear collars. Additionally, concern regarding the stress experienced by the grizzly bears between the time of capture and time of handling is lessened as activation alarms are added to traps. These technological advances could provide greater insight into the daily experiences of grizzly bears and reduce the duration of stress imposed by capture.

The custom microarray developed in this study evaluated protein expression in the skin of grizzly bears from Western Alberta and found patterns of protein expression that correlated with assessments of health, such as body length and mass, and landscape measurements, such as the anthropogenic change and road density in the landscape. The challenge with all biomarkers is evaluating linkages between biomarker responses and adverse effects on individual fitness and

population dynamics. Protein expression changes in skin may provide wildlife managers with another indicator of potential allostatic load or reactive scope to utilize in making decisions regarding wildlife conservation and potential impacts of landscape alterations.

**Literature Cited**

Aich P, Jalal S, Czuba C, Schatte G, Herzog K, Olson DJ, Ross AR, Potter AA, Babiuk LA, Griebel P. 2007. Comparative approaches to the investigation of responses to stress and viral infection in cattle. Omics 11(4): 413-34.

Aizawa K, Nakahori C, Akimoto T, Kimura F, Hayashi K, Kono I, Mesaki N. 2006. Changes of pituitary, adrenal and gonadal hormones during competition among female soccer players. J Sports Med Phys Fitness 46(2): 322-7.

Aktan F. 2004. iNOS-mediated nitric oxide production and its regulation. Life Sci 75(6): 639-53.

Alberta Grizzly Bear Recovery Team. 2008. Alberta grizzly bear recovery plan 2008-2013. Edmonton, AB: Alberta Sustainable Resource Development, Fish and Wildlife Division. Report nr Alberta species at risk recovery plan No. 15. 68 p.

Alberta Sustainable Resource Development. 2006. The general status of Alberta wild species 2005.

Alberta Sustainable Resource Development and Alberta Conservation Association. 2010. Status of the Grizzly Bear (*Ursus arctos*) in Alberta: Update 2010. Edmonton, AB: Alberta Sustainable Resource Development. Report nr Wildlife Status Report No. 37. 44 p.

Alibhai SK, Jewell ZC, Towindo SS. 2001. Effects of immobilization on fertility in female black rhino (*Diceros bicornis*). J Zool Lond 253: 333-345.

American Society of Mammalogists Animal Care and Use Committee. 1998. American Society of Mammalogists Animal Care and Use Committee Guidelines. American Society of Mammalogists.

Angelier F, Chastel O. 2009. Stress, prolactin and parental investment in birds: a review. Gen Comp Endocrinol 163(1-2):142-8.

Aoyagi T, Koshimizu TA, Tanoue A. 2009. Vasopressin regulation of blood pressure and volume: findings from V1a receptor-deficient mice. Kidney Int 76(10): 1035-9.

Arck PC, Slominski A, Theoharides TC, Peters EM, Paus R. 2006. Neuroimmunology of stress: skin takes center stage. J Invest Dermatol 126(8): 1697-704.

Arlettaz R, Patthey P, Baltic M, Leu T, Schaub M, Palme R, Jenni-Eiermann S. 2007. Spreading free-riding snow sports represent a novel serious threat for wildlife. Proc Biol Sci 274(1614): 1219-24.

Armario A. 2006. The hypothalamic-pituitary-adrenal axis: what can it tell us about stressors?

CNS Neurol Disord Drug Targets 5(5): 485-501.

Arrigo AP. 2001. Hsp27: novel regulator of intracellular redox state. IUBMB Life 52(6): 303-7.

Arya R, Mallik M, Lakhotia SC. 2007. Heat shock genes - integrating cell survival and death. J Biosci 32(3): 595-610.

auf dem Keller U, Kumin A, Braun S, Werner S. 2006. Reactive oxygen species and their detoxification in healing skin wounds. J Investig Dermatol Symp Proc 11(1): 106-11.

Balistreri CR, Caruso C, Grimaldi MP, Listi F, Vasto S, Orlando V, Campagna AM, Lio D, Candore G. 2007. CCR5 receptor: biologic and genetic implications in age-related diseases. Ann N Y Acad Sci 1100: 162-72.

Barclay D, Zamora R, Torres A, Namas R, Steed D, Vodovotz Y. 2008. A simple, rapid, and convenient Luminex-compatible method of tissue isolation. J Clin Lab Anal 22(4): 278-81.

Barnes TS, Goldizen AW, Morton JM, Coleman GT. 2010. Parasites of the brush-tailed rock-wallaby (*Petrogale penicillata*). J Wildl Dis 46(1): 218-28.

Barrier M, Mirkes PE. 2005. Proteomics in developmental toxicology. Reprod Toxicol 19(3): 291-304.

Bechert US, Southern SO. Monitoring environmental stress in African elephants (*Loxodonta africana*) through molecular analysis of stress-activated proteins; 2002. p 249-253.

Beckwitt R, Shea J, Osborne D, Krueger S, Barklow W. 2002. A PCR-based method for sex identification in Hippopotamus amphibius. Afr Zool 37(2):127-130.

Benn B, Herrero S. 2002. Grizzly bear mortality and human access in Banff and Yoho national parks, 1971-98. Ursus 13: 213-221.

Berland A, Nelson T, Stenhouse G, Graham K, Cranston J. 2008. The impact of landscape disturbance on grizzly bear habitat use in the Foothills Model Forest, Alberta, Canada. For Ecol Mange 256(11): 1875-1883.

Berlier JE, Rothe A, Buller G, Bradford J, Gray DR, Filanoski BJ, Telford WG, Yue S, Liu J, Cheung CY and others. 2003. Quantitative comparison of long-wavelength Alexa Fluor dyes to Cy dyes: fluorescence of the dyes and their bioconjugates. J Histochem Cytochem 51(12): 1699-712.

Berry MD, Boulton AA. 2000. Glyceraldehyde-3-phosphate dehydrogenase and apoptosis. J Neurosci Res 60: 150-154.

Bird CE, Seeman TE, Escarce JJ, Basurto-Davila R, Finch BK, Dubowitz T, Heron M, Hale L, Merkin SS, Weden M and others. 2009. Neighborhood Socioeconomic Status and Biological "Wear & Tear" in a Nationally Representative Sample of U.S. Adults. J Epidemiol Community Health doi:10.1136/jech.2008.084814:1-19.

Bjarnadottir SG, Hollung K, Faergestad EM, Veiseth-Kent E. 2010. Proteome changes in bovine longissimus thoracis muscle during the first 48 h postmortem: shifts in energy status and myofibrillar stability. J Agric Food Chem 58(12): 7408-14.

Black PH. 2002. Stress and the inflammatory response: a review of neurogenic inflammation. Brain Behav Immun 16(6): 622-53.

Black PH, Garbutt LD. 2002. Stress, inflammation and cardiovascular disease. J Psychosom Res 52(1): 1-23.

Bonier F, Martin PR, Moore IT, Wingfield JC. 2009. Do baseline glucocorticoids predict fitness? Trends Ecol Evol 24(11): 634-42.

Boonstra R. 2005. Equipped for life: the adaptive role of the stress axis in male mammals. J Mammal 86(2): 236-247.

Boonstra R, Hik K, Singleton GR, Tinnikov A. 1998. The impact of predator-induced stress on the snowshoe hare cycle. Ecol Monograph 79(5): 371-394.

Boonstra R, McColl CJ. 2000. Contrasting stress response of male arctic ground squirrels and red squirrels. J Exp Zool 286(4): 390-404.

Boulais N, Misery L. 2008. The epidermis: a sensory tissue. Eur J Dermatol 18(2): 119-27.

Boulanger J, Cranston J, Nielsen S, Stenhouse GB. 2009. Estimation of grizzly bear population size for the Swan Hills management unit using DNA sampling and habitat-relative occupancy models. Alberta Sustainable Resource Development. 16 p.

Boyce MS. 2006. Scale for resource selection functions. Diversity Distrib 12: 269-276.

Boyce MS, McDonald LL. 1999. Relating populations to habitats using resource selection functions. Trend Ecol Evol 12: 269-276.

Boyce MS, Nielsen S, Stenhouse G. 2009. Grizzly bears benefit from forestry - except for the roads. BC Forest Prof. 16(1):19.

Breloer M, Dorner B, More SH, Roderian T, Fleischer B, von Bonin A. 2001. Heat shock proteins as "danger signals": eukaryotic Hsp60 enhances and accelerates antigen-specific IFN-gamma production in T cells. Eur J Immunol 31(7): 2051-9.

Brown DE. 1985. The grizzly in the southwest: University of Oklahoma Press.

Cahill DJ. 2001. Protein and antibody arrays and their medical applications. J Immunol Methods 250(1-2): 81-91.

Calderwood SK, Mambula SS, Gray PJ, Jr., Theriault JR. 2007. Extracellular heat shock proteins in cell signaling. FEBS Lett 581(19): 3689-94.

Cals-Grierson MM, Ormerod AD. 2004. Nitric oxide function in the skin. Nitric Oxide 10(4): 179-93.

Campisi J, Leem TH, Fleshner M. 2003. Stress-induced extracellular Hsp72 is a functionally significant danger signal to the immune system. Cell Stress Chaperones 8(3): 272-86.

Canadian Council on Animal Care. 2003. CCAC guidelines on the care and use of wildlife. Ottawa, Ontario: Canadian Council on Animal Care. 66 p.

Cande C, Cecconi F, Dessen P, Kroemer G. 2002. Apoptosis-inducing factor (AIF): key to the conserved caspase-independent pathways of cell death? J Cell Sci 115(Pt 24): 4727-34.

Cannon WB. 1914. The emergency function of the adrenal medulla in pain and the major emotions. Am J Physiol 33(2): 356-372.

Cardillo M, Mace GM, Gittleman JL, Purvis A. 2006. Latent extinction risk and the future battlegrounds of mammal conservation. Proc Natl Acad Sci U S A 103(11): 4157-61.

Cardillo M, Mace GM, Jones KE, Bielby J, Bininda-Emonds ORP, Sechrest W, Orme CDL, Purvis A. 2005. Multiple causes of high extinction risk in large mammal species. Science 309.

Cardillo M, Purvis A, Sechrest W, Gittleman JL, Bielby J, Mace GM. 2004. Human population density and extinction risk in the world's carnivores. PLoS Biol 2(7): 0909-0914.

Carrasco GA, Van de Kar LD. 2003. Neuroendocrine pharmacology of stress. Eur J Pharmacol 463(1-3): 235-72.

Carroll C. 2007. Interacting effects of climate change, landscape conversion, and harvest on carnivore populations at the Range mountain: Marten and lynx in the Northern Appalachians. Conserv Biol 21(4): 1092-1104.

Cattet M. 2008. Foothills model forest grizzly bear research program 2008: Protocol for the capture, handling, and sampling of grizzly bears. Saskatoon: Canadian Cooperative Wildlife Health Center. 17 p.

Cattet M, Boulanger J, Stenhouse G, Powell RA, Reynolds-Hogland MJ. 2008a. An evaluation

of long-term capture effects in ursids: implications for wildlife welfare and research. J Mamm 89(4): 973-990.

Cattet M, Stenhouse G, Bollinger T. 2008b. Exertional myopathy in a grizzly bear (*Ursus arctos*) captured by leghold snare. J Wildl Dis 44(4): 973-8.

Cattet M, Vijayan M, Janz D. 2008c. Wildlife Health. In: Stenhouse G, Graham K, editors. Foothills research institute grizzly bear program 2008 annual report. Hinton, AB.

Cattet MRL, Caulkett NA, Obbard ME, Stenhouse GB. 2002. A body-condition index for ursids. Can J Zool 80: 1156-1161.

Cattet MRL, Christison K, Caulkett NA, Stenhouse GB. 2003. Physiologic responses of grizzly bears to different methods of capture. J Wildl Dis 39(3): 649-654.

Chandra D, Choy G, Tang DG. 2007. Cytosolic accumulation of HSP60 during apoptosis with or without apparent mitochondrial release: evidence that its pro-apoptotic or pro-survival functions involve differential interactions with caspase-3. J Biol Chem 282(43): 31289-301.

Chang TS, Cho CS, Park S, Yu S, Kang SW, Rhee SG. 2004. Peroxiredoxin III, a mitochondrion-specific peroxidase, regulates apoptotic signaling by mitochondria. J Biol Chem 279(40): 41975-84.

Chao HM, Blanchard DC, Blanchard RJ, McEwen BS, Sakai RR. 1993. The effect of social stress on hippocampal gene expression. Mol Cell Neurosci 4: 543-548.

Chapman BP, Moynihan J. 2009. The brain-skin connection: role of psychosocial factors and neuropeptides in psoriasis. Expert Rev Clin Immunol 5(6): 623-7.

Chapman CA, Lambert JE. 2000. Habitat alteration and the conservation of African primates: case study of Kibale National Park, Uganda. Am J Prim 50(3): 169-185.

Charmandari E, Tsigos C, Chrousos G. 2005. Endocrinology of the stress response. Annu Rev Physiol 67: 259-84.

Charveron M, Calvo M, Gall Y. 1995. Cell stress and implications of the heat-shock response in skin. Cell Biol Toxicol 11(3-4): 161-5.

Chen Z, Xu H, Haimano S, Li X, Li XM. 2005. Quetiapine and venlafaxine synergically regulate heme oxygenase-2 protein expression in the hippocampus of stressed rats. Neurosci Lett 389(3): 173-7.

Cherel Y, Mauget R, Lacroix A, Gilles J. 1994. Seasonal and fasting-related changes in

circulating gonadal steroids and prolactin in king penguins, Aptenodytes patagonicus. Physiol Biochem Zool 67(5): 1154-1173.

Choudhury SD, Allsop T, Passman A, Norris GE. 2006. Use of a proteomics approach to identify favorable conditions for production of good quality lambskin leather. Anal Bioanal Chem 384(3): 723-35.

Chrousos GP, Gold PW. 1992. The concepts of stress and stress system disorders. Overview of physical and behavioural homeostasis. Jama 267(9): 1244-52.

Chuang DM, Hough C, Senatorov VV. 2005. Glyceraldehyde-3-phosphate dehydrogenase, apoptosis, and neurodegenerative diseases. Annu Rev Pharmacol Toxicol 45: 269-90.

Ciarniello LM, Boyce MS, Heard DC, Seip DR. 2007. Components of grizzly bear habitat selection: density, habitats, roads, and mortality risk. J Wildl Manage 71(5): 1446-1457.

Ciarniello LM, Boyce MS, Seip DR, Heard DC. 2009. Comparison of grizzly bear Ursus arctos demographics in wilderness mountains versus a plateau with resource development. Wildl Biol 15(3): 247-265.

Clark JS, Carpenter SR, Barber M, Collins S, Dobson A, Foley JA, Lodge DM, Pascual M, Pielke R, Jr., Pizer W and others. 2001. Ecological forecasts: an emerging imperative. Science 293(5530): 657-60.

Clincy M, Krebs CJ, Jarman PJ. 2001. Dispersal sinks and handling effects: interpreting the role of immigration in common brushtail possum populations. J Anim Ecol 70: 515-526.

Clubb R, Mason G. 2003. Animal welfare: captivity effects on wide-ranging carnivores. Nature 425(6957): 473-4.

Cohen GM. 1997. Caspases: the executioners of apoptosis. Biochem J 326 ( Pt 1): 1-16.

Concannon CG, Gorman AM, Samali A. 2003. On the role of Hsp27 in regulating apoptosis. Apoptosis 8(1): 61-70.

Cooke SJ, Cory DS. 2008. Ecological restoration and physiology: an overdue integration. BioScience 58(10): 957-968.

Cooke SJ, O'Connor CM. 2010. Making conservation physiology relevant to policy makers and conservation practitioners. Conserv Lett 3: 159-166.

Copeland JP. 1996. Biology of the wolverine in Central Idaho. Moscow: University of Idaho. 138 p.

Cote SD, Festa-Bianchet M, Fournier F. 1998. Life-history effects of chemical immobilization

and radiocollars on mountain goats. J Wildl Manage 62(2): 745-752.

Creel S, Creel NM, Monfort SL. 1997. Radiocollaring and stress hormones in African wild dogs. Conserv Biol 11(2): 544-548.

Creel S, Fox JE, Hardy A, Sands J, Garrott B, Peterson RO. 2002. Snowmobile activity and glucocorticoid stress responses in wolves and elk. Conserv Biol 16(3): 809-814.

Curry BE. 1999. The potential influence of fishery-induced stress on dolphins in the eastern tropical pacific ocean. International dolphin conservation program act. Report nr NOAA technical memorandum 260. 1-53 p.

Curry BE, Edwards EF. 1998. Investigation of the potential influence of fishery-induced stress on dolphins in the Eastern Tropical Pacific Ocean: Research Planning. National Marine Fisheries Service, NOAA. Report nr NOAA-TM-NMFS-SWFSC-254. 1-59 p.

Cutler P. 2003. Protein arrays: the current state-of-the-art. Proteomics 3(1): 3-18.

Cyr NE, Michael Romero L. 2007. Chronic stress in free-living European starlings reduces corticosterone concentrations and reproductive success. Gen Comp Endocrinol 151(1): 82-9.

Das A, Rai D, Dikshit M, Palit G, Nath C. 2005. Nature of stress: differential effects on brain acetylcholinesterase activity and memory in rats. Life Sci 77(18): 2299-311.

de Castro AB, Voss JG, Ruppin A, Dominguez CF, Seixas NS. 2010. Stressors among Latino day labourers. A pilot study examining allostatic load. Aaohn J 58(5): 185-96.

de Kloet ER. 2003. Hormones, brain and stress. Endocr Regul 37(2): 51-68.

Deem SI, Karesh WB, Weisman W. 2001. Putting theory into practice: wildlife health in conservation. Conserv Biol 15(5): 1224-1233.

Degterev A, Boyce M, Yuan J. 2003. A decade of caspases. Oncogene 22(53): 8543-67.

Dhabhar FS. 2000. Acute stress enhances while chronic stress suppresses skin immunity. The role of stress hormones and leukocyte trafficking. Ann N Y Acad Sci 917: 876-93.

Dickens MJ, Delehanty DJ, Romero LM. 2009. Stress and translocation: alterations in the stress physiology of translocated birds. Proc Biol Sci 276(1664): 2051-6.

Dickens MJ, Delehanty DJ, Romero LM. 2010. Stress: An inevitable component of animal translocation. Biol Cons 143: 1329-1341.

Dizon A, Allen A, Kellar N, Southern S. 2002. Stress in spotted dolphins (*Stenella attenuata*) associated with purse-seine tuna fishing in the Eastern tropical Pacific. La Jolla:

Southwest Fisheries Sciences Center. Report nr LJ-02-26. 23 p.

Donaldson ZR, Young LJ. 2008. Oxytocin, vasopressin, and the neurogenetics of sociality. Science 322(5903): 900-4.

Doran P, Dowling P, Lohan J, McDonnell K, Poetsch S, Ohlendieck K. 2004. Subproteomics analysis of Ca+-binding proteins demonstrates decreased calsequestrin expression in dystrophic mouse skeletal muscle. Eur J Biochem 271(19): 3943-52.

dos Santos Schmidt EM, Paulillo AC, Lapera IM, Vieira Martins GR, Junior LN, Testi AJP. 2010. Serum biochemical parameters of female bronze turkeys (*Meleagris gallopavo*) during egg-laying season. Int J Poult Sci 9(2): 177-179.

Dostert A, Heinzel T. 2004. Negative glucocorticoid receptor response elements and their role in glucocorticoid action. Curr Pharm Des 10(23): 2807-16.

Dragovic Z, Broadley SA, Shomura Y, Bracher A, Hartl FU. 2006. Molecular chaperones of the Hsp110 family act as nucleotide exchange factors of Hsp70s. Embo J 25(11): 2519-28.

Dronjak S, Gavrilovic L, Filipovic D, Radojcic MB. 2004. Immobilization and cold stress affect sympatho-adrenomedullary system and pituitary-adrenocortical axis of rats exposed to long-term isolation and crowding. Physiol Behav 81(3): 409-15.

Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB, Lipsky PE. 1998. Cyclooxygenase in biology and disease. Faseb J 12(12): 1063-73.

Duclos M, Martin C, Malgat M, Mazat JP, Chaouloff F, Mormede P, Letellier T. 2001. Relationships between muscle mitochondrial metabolism and stress-induced corticosterone variations in rats. Pflugers Arch 443(2): 218-26.

Durackova Z. 2010. Some current insights into oxidative stress. Physiol Res 59: 459-469.

Easton DP, Kaneko Y, Subjeck JR. 2000. The hsp110 and Grp1 70 stress proteins: newly recognized relatives of the Hsp70s. Cell Stress Chaperones 5(4): 276-90.

Espina V, Mehta AI, Winters ME, Calvert V, Wulfkuhle J, Petricoin EF, 3rd, Liotta LA. 2003. Protein microarrays: molecular profiling technologies for clinical specimens. Proteomics 3(11): 2091-100.

Evans GW, Schamberg MA. 2009. Childhood poverty, chronic stress, and adult working memory. Proc Natl Acad Sci U S A.

Evers AW, Verhoeven EW, Kraaimaat FW, de Jong EM, de Brouwer SJ, Schalkwijk J, Sweep FC, van de Kerkhof PC. 2010. How stress gets under the skin: cortisol and stress

reactivity in psoriasis. Br J Dermatol 163(5): 986-91.

Fahrig L, Rytwinski T. 2009. Effects of roads on animal abundance: an empirical review and synthesis. Ecol Soc 14(1): 21.

Fan CY, Lee S, Ren HY, Cyr DM. 2004. Exchangeable chaperone modules contribute to specification of type I and type II Hsp40 cellular function. Mol Biol Cell 15(2): 761-73.

Feder ME, Hofmann GE. 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 61: 243-82.

Findlay CS, Bourdages J. 2000. Response time of wetland biodiversity to road construction on adjacent lands. Conserv Biol 14(1): 86-94.

Fleshner M, Campisi J, Amiri L, Diamond DM. 2004. Cat exposure induces both intra- and extracellular Hsp72: the role of adrenal hormones. Psychoneuroendocrinology 29(9): 1142-52.

Forley SD, Robbins CT. 1995. Lactation, hibernation, and mass dynamics of American black bears and grizzly bears. Can J Zool 73(12): 2216-2222.

Forman RTT, Alexander L. 1998. Roads and their major ecological effects. Annu Rev Ecol Syst 29: 207-31.

Forney KA, St. Aubin DJ, Chivers SJ. 2002. Chase encirclement stress studies on dolphins involved in Eastern tropical Pacific Ocean purse-seine operations during 2001. La Jolla: Southwest Fisheries Science Center. Report nr LJ-02-32. 1-26 p.

Forstermann U. 2006. Endothelial NO synthase as a source of NO and superoxide. Eur J Clin Pharmacol 62 Suppl 13: 5-12.

Furukawa F, Fujii K, Horiguchi Y, Matsuyoshi N, Fujita M, Toda K, Imamura S, Wakita H, Shirahama S, Takigawa M. 1997. Roles of E- and P-cadherin in the human skin. Microsc Res Tech 38(4): 343-52.

Galindo RC, Ayoubi P, Naranjo V, Gortazar C, Kocan KM, de la Fuente J. 2009. Gene expression profiles of European wild boar naturally infected with Mycobacterium bovis. Vet Immunol Immunopathol 129(1-2): 119-25.

Garrido C, Gurbuxani S, Ravagnan L, Kroemer G. 2001. Heat shock proteins: endogenous modulators of apoptotic cell death. Biochem Biophys Res Commun 286(3): 433-42.

Garshelis DL. 2002. Misconceptions, ironies, and uncertainties regarding trends in bear populations. Ursus 13: 321-334.

Garshelis DL, Gibeau ML, Herrero S. 2005. Grizzly bear demographics in and around Banff National Park and Kananaskis Country, Alberta. J Wildl Manage 69(1): 277-297.

Gerke V, Creutz CE, Moss SE. 2005. Annexins: linking Ca2+ signalling to membrane dynamics. Nat Rev Mol Cell Biol 6(6): 449-61.

Gerke V, Moss SE. 2002. Annexins: from structure to function. Physiol Rev 82(2): 331-71.

Gerthoffer WT, Gunst SJ. 2001. Signal transduction in smooth muscle: Focal adhesion and small heat shock proteins in the regulation of actin remodeling and contractility in smooth muscle. J Appl Physiol 91: 963-972.

Gething MJ, Sambrook J. 1992. Protein folding in the cell. Nature 355(6355): 33-45.

Gibeau ML, Clevenger AP, Herrero S, Wierzchowski J. 2002. Grizzly bear response to human development and activities in the Bow River Watershed, Alberta Canada. Biol Conserv 103: 227-236.

Girault J, Gorelick FS, Greengard P. 1989. Improving the quality of immunoblots by chromatography of polyclonal antisera on keratin affinity columns. Anal Biochem 182(1): 193-196.

Glaser R, Kiecolt-Glaser JK. 2005. Stress-induced immune dysfunction: implications for health. Nat Rev 5: 243-251.

Government of Alberta. 2010. Grizzly bear conservation in Alberta: 2009 management activities and recovery implementation. Report nr I/445.

Graham K, Boulanger J, Duval J, Stenhouse G. 2010. Spatial and temporal use of roads by grizzly bears in west-central Alberta. Ursus 21(1): 43-56.

Green J, Pacas C, Bayley S, Cornwell L. 1996. Ecological outlooks project. A cumulative effects assessment and futures outlook of the Banff Bow Valley. Ottawa, ON: Banff Bow Valley Study. Department of Canadian Heritage.

Guzik TJ, Korbut R, Adamek-Guzik T. 2003. Nitric oxide and superoxide in inflammation and immune regulation. J Physiol Pharmacol 54(4): 469-87.

Haab BB. 2001. Advances in protein microarray technology for protein expression and interaction profiling. Curr Opin Drug Discov Devel 4(1): 116-23.

Haab BB. 2003. Methods and applications of antibody microarrays in cancer research. Proteomics 3(11): 2116-22.

Haab BB, Dunham MJ, Brown PO. 2001. Protein microarrays for highly parallel detection and

quantitation of specific proteins and antibodies in complex solutions. Genome Biol 2(2):RESEARCH0004.

Haab BB, Zhou Z. 2004. Multiplexed protein analysis using spotted antibody microarrays. In: Fung E, editor. Protein arrays: methods and protocols. Totowa, NJ: Humana Press. p 33-45.

Hamann B, Johnston H, Gobielle J, Hillis M, Johnson S, Kelly L, McClelland P. 1999. Birds. In: Joslin G, Youmans H, editors. Effects of recreation on Rocky Mountain Wildlife: A review for Montana: Committee on Effects of Recreation on Wildlife, Montana Chapter of The Wildlife Society. 34 p.

Hamer D. 1996a. Buffaloberry (*Shepherdia canadensis*) fruit production in fire-successional bear feeding sites. Banff National Park, Banff, AB. 59 p.

Hamer D. 1996b. Wildfire's influence on Yellow Hedysarum digging habitat used by grizzly bears in Banff National Park, Alberta: Summary of results from the 1995 field season. Banff National Park. Banff, AB. 13 p.

Hamer D, Herrero S. 1983. Ecological studies of the grizzly bear in Banff National Park: final report. Calgary, Alberta: Parks Canada. 303 p.

Hamer D, Herrero S. Wildfire's influence on bear feeding ecology in Banff National Park, Alberta. In: P. Z, Beecham J, Matula GJ, Reynolds H, editors; 1987; Williamsburg, VA. International Association for Bear Research and Management. p 179-186.

Hamilton JW. 2007. Evaluation of indicators of stress in populations of polar bears (*Ursus maritimus*) and grizzly bears (*Ursus arctos*). Waterloo, Ontario: University of Waterloo. 92 p.

Hampson SE, Goldberg LR, Vogt TM, Hillier TA, Dubanoski JP. 2009. Using physiological dysregulation to assess global health status: associations with self-rated health and health behaviours. J Health Psychol 14(2): 232-41.

Hara MR, Cascio MB, Sawa A. 2006. GAPDH as a sensor of NO stress. Biochim Biophys Acta 1762(5): 502-9.

Harris RB, Mitchell TD, Simpson J, Redmann SM, Jr., Youngblood BD, Ryan DH. 2002. Weight loss in rats exposed to repeated acute restraint stress is independent of energy or leptin status. Am J Physiol Regul Integr Comp Physiol 282(1): R77-88.

Heath JA, Dufty AM, Jr. 1998. Body condition and the adrenal stress response in captive

American kestrel juveniles. Physiol Zool 71(1): 67-73.

Heckmann LH, Sibly RM, Connon R, Hooper HL, Hutchinson TH, Maund SJ, Hill CJ, Bouetard A, Callaghan A. 2008. Systems biology meets stress ecology: linking molecular and organismal stress responses in Daphnia magna. Genome Biol 9(2): R40.

Henderson B. 2010. Integrating the cell stress response: a new view of molecular chaperones as immunological and physiological homeostatic regulators. Cell Biochem Funct 28:1-14.

Hengartner MO. 2000. The biochemistry of apoptosis. Nature 407(6805): 770-6.

Herman JP, Figueiredo H, Mueller NK, Ulrich-Lai Y, Ostrander MM, Choi DC, Cullinan WE. 2003. Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. Front Neuroendocrinol 24(3): 151-80.

Hinz B, Brune K. 2002. Cyclooxygenase-2--10 years later. J Pharmacol Exp Ther 300(2):367-75.

Hobson D. 2006. Summary of 2005 spring capture program. In: Stenhouse G, Graham K, editors. Foothills Model Forest Grizzly Bear Research Program 2005 Annual Report. Hinton, Alberta. p 3-8.

Hobson D, Cranston J, Stenhouse G, Larsen T, Pigeon K. 2008. Program field activities 2007. In: Stenhouse G, Graham K, editors. Foothills Model Forest Grizzly Bear Research Program Annual report 2007. Hinton, Alberta: Foothills Model Forest. p 3-13.

Homan RN, Regosin JV, Rodrigues DM, Reed JM, Windmiller BS, Romero LM. 2003. Impacts of varying habitat quality on the physiological stress of spotted salamanders (*Ambystoma maculatum*). Anim Cons 6: 11-18.

Hornocker MG, Hash HS. 1981. Ecology of the wolverine in Northwestern Montana. Can J Zool 59:1286-1301.

Hosoi J. 2006. Stress and the skin. Int J Cosmetic Sci 28: 243-246.

Ito N, Ito T, Kromminga A, Bettermann A, Takigawa M, Kees F, Straub RH, Paus R. 2005. Human hair follicles display a functional equivalent of the hypothalamic-pituitary-adrenal axis and synthesize cortisol. Faseb J 19(10): 1332-4.

IUCN. 2010. Guidelines for using the IUCN red list categories and criteria. Version 8.1. Prepared by the Standards and Petitions Subcommittee.

Jeon YJ, Kim DH, Jung H, Chung SJ, Chi SW, Cho S, Lee SC, Park BC, Park SG, Bae KH. 2010. Annexin A4 interacts with the NF-kappaB p50 subunit and modulates NF-kappaB

transcriptional activity in a Ca(2+)-dependent manner. Cell Mol Life Sci DOI 10.1007/s00018-010-0331-9.

Jia X, Ekman M, Grove H, Faergestad EM, Aass L, Hildrum KI, Hollung K. 2007. Proteome changes in bovine longissimus thoracis muscle during the early postmortem storage period. J Proteome Res 6(7): 2720-31.

Johnson CJ, Boyce MS, Case RL, Cluff HD, Gau RJ, Gunn A, Mulders R. 2005. Cumulative effects of human developments on arctic wildlife. Wildl Monogr 160: 1-36.

Johnson CJ, Seip DR. 2008. Relationship between resource selection, distribution, and abundance: a test with implications to theory and conservation. Popul Ecol 50(2): 145-157.

Kalmar B, Greensmith L. 2009. Induction of heat shock proteins for protection against oxidative stress. Adv Drug Deliv Rev 61(4): 310-8.

Kennerly E, Ballmann A, Martin S, Wolfinger R, Gregory S, Stoskopf M, Gibson G. 2008. A gene expression signature of confinement in peripheral blood of red wolves (*Canis rufus*). Mol Ecol 17(11): 2782-91.

Kleinert H, Pautz A, Linker K, Schwarz PM. 2004. Regulation of the expression of inducible nitric oxide synthase. Eur J Pharmacol 500(1-3): 255-66.

Kokoszka JE, Coskun P, Esposito LA, Wallace DC. 2001. Increased mitochondrial oxidative stress in the Sod2 (+/-) mouse results in the age-related decline of mitochondrial function culminating in increased apoptosis. Proc Natl Acad Sci U S A 98(5): 2278-83.

Konig S, Luger TA, Scholzen TE. 2006. Monitoring neuropeptide-specific proteases: processing of the proopiomelanocortin peptides adrenocorticotropin and alpha-melanocyte-stimulating hormone in the skin. Exp Dermatol 15(10): 751-61.

Kono M, Nagata H, Umemura S, Kawana S, Osamura RY. 2001. In situ expression of corticotropin-releasing hormone (CRH) and proopiomelanocortin (POMC) genes in human skin. Faseb J 15(12): 2297-9.

Kopecek P, Altmannova K, Weigl E. 2001. Stress proteins: nomenclature, division and functions. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 145(2): 39-47.

Krantic S, Mechawar N, Reix S, Quirion R. 2007. Apoptosis-inducing factor: a matter of neuron life and death. Prog Neurobiol 81(3): 179-96.

Kreeger TJ, White PJ, Seal US, Tester JR. 1990. Pathological responses of red foxes to foothold

traps. J Wildl Manage 54(1): 147-159.

Kregel KC. 2002. Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. J Appl Physiol 92(5): 2177-86.

Kultz D. 2005. Molecular and evolutionary basis of the cellular stress response. Annu Rev Physiol 67: 225-57.

Kunzl C, Sachser N. 1999. The behavioural endocrinology of domestication: A comparison between the domestic guinea pig (*Cavia aperea f. porcellus*) and its wild ancestor, the cavy (*Cavia aperea*). Horm Behav 35(1): 28-37.

Lamkanfi M, Declercq W, Kalai M, Saelens X, Vandenabeele P. 2002. Alice in caspase land. A phylogenetic analysis of caspases from worm to man. Cell Death Differ 9(4): 358-61.

Lamkanfi M, Kanneganti TD, Franchi L, Nunez G. 2007. Caspase-1 inflammasomes in infection and inflammation. J Leukoc Biol 82(2): 220-5.

Lee DH, Blomhoff R, Jacobs DR, Jr. 2004. Is serum gamma glutamyltransferase a marker of oxidative stress? Free Radic Res 38(6): 535-9.

Leonard BE. 2005. The HPA and immune axes in stress: the involvement of the serotonergic system. Eur Psychiatry 20 Suppl 3: S302-6.

Lewthwaite J, Owen N, Coates A, Henderson B, Steptoe A. 2002. Circulating human heat shock protein 60 in the plasma of British civil servants: relationship to physiological and psychosocial stress. Circulation 106(2): 196-201.

Lindsjo HJA. 2009. Development and application of a health function score system for grizzly bears (*Ursus arctos*) in western Alberta. Saskatoon: University of Saskatchewan.

Liotta LA, Espina V, Mehta AI, Calvert V, Rosenblatt K, Geho D, Munson PJ, Young L, Wulfkuhle J, Petricoin EF, 3rd. 2003. Protein microarrays: meeting analytical challenges for clinical applications. Cancer Cell 3(4): 317-25.

Liu XH, Qian LJ, Gong JB, Shen J, Zhang XM, Qian XH. 2004. Proteomic analysis of mitochondrial proteins in cardiomyocytes from chronic stressed rat. Proteomics 4(10): 3167-76.

Liu Y, Steinacker JM. 2001. Changes in skeletal muscle heat shock proteins: pathological significance. Front Biosci 6: D12-25.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the folin phenol reagent. J Biol Chem 193: 265-275.

Lundberg E, Fagerberg L, Klevebring D, Matic I, Geiger T, Cox J, Algenas C, Lundeberg J, Mann M, Uhlen M. 2010. Defining the transcriptome and proteome in three functionally different human cell lines. Mol Syst Biol 6: 450.

MacArthur RA, Geist V, Johnson RH. 1982. Cardiac and behavioural responses of mountain sheep to human disturbance. J Wildl Manage 46: 351-358.

MacBeath G. 2002. Protein microarrays and proteomics. Nat Genet 32 Suppl: 526-32.

Macbeth BJ, Cattet MRL, Stenhouse GB, Gibeau ML, Janz DM. 2010. Hair cortisol concentration as a non-invasive measure of long-term stress in free-ranging grizzly bears (*Ursus arctos*): technique development and considerations with implications for other wildlife. Can J Zool 88(10): 935-949.

Madrigal JL, Olivenza R, Moro MA, Lizasoain I, Lorenzo P, Rodrigo J, Leza JC. 2001. Glutathione depletion, lipid peroxidation and mitochondrial dysfunction are induced by chronic stress in rat brain. Neuropsychopharmacology 24(4): 420-9.

Magin TM, Vijayaraj P, Leube RE. 2007. Structural and regulatory functions of keratins. Exp Cell Res 313(10): 2021-32.

Maines MD. 1997. The heme oxygenase system: a regulator of second messenger gases. Annu Rev Pharmacol Toxicol 37: 517-54.

Maines MD, Panahian N. 2001. The heme oxygenase system and cellular defense mechanisms. In: Roach RC, Wagner PD, Hackett PH, editors. Hypoxia from genes to the bedside. New York: Kluwer Academic/Plenum Publishers.

Malarkey WB, Kiecolt-Glaser JK, Pearl D, Glaser R. 1994. Hostile behaviour during marital conflict alters pituitary and adrenal hormones. Psychosom Med 56(1): 41-51.

Mancia A, Warr GW, Chapman RW. 2008. A transcriptomic analysis of the stress induced by capture-release health assessment studies in wild dolphins (*Tursiops truncatus*). Mol Ecol 17(11): 2581-9.

Manoli I, Alesci S, Blackman MR, Su YA, Rennert OM, Chrousos GP. 2007. Mitochondria as key components of the stress response. Trends Endocrinol Metab 18(5): 190-8.

Marques AH, Silverman MN, Sternberg EM. 2010. Evaluation of stress systems by applying noninvasive methodologies: measurements of neuroimmune biomarkers in the sweat, heart rate variability and salivary cortisol. Neuroimmunomodulation 17(3): 205-8.

Martin B, Golden E, Carlson OD, Egan JM, Mattson MP, Maudsley S. 2008. Caloric restriction:

impact upon pituitary function and reproduction. Ageing Res Rev 7(3): 209-24.

Martinez-Mota R, Valdespino C, Sanchez-Ramos MA, Serio-Silva JC. 2007. Effects of forest fragmentation on the physiological stress response of black howler monkeys. Anim Cons 10: 374-379.

Martiny JB, Bohannan BJ, Brown JH, Colwell RK, Fuhrman JA, Green JL, Horner-Devine MC, Kane M, Krumins JA, Kuske CR and others. 2006. Microbial biogeography: putting microorganisms on the map. Nat Rev Microbiol 4(2): 102-12.

Mason GJ, Veasey JS. 2010. How should the psychological well-being of zoo elephants be objectively investigated? Zoo Biol 29(2): 237-55.

Mastorakos G, Pavlatou M, Diamanti-Kandarakis E, Chrousos GP. 2005. Exercise and the stress system. Hormones (Athens) 4(2): 73-89.

Mattei J, Demissie S, Falcon LM, Ordovas JM, Tucker K. 2010. Allostatic load is associated with chronic conditions in the Boston Puerto Rican Health Study. Soc Sci Med 70(12): 1988-96.

Mattson DJ, Herrero S, Wright RG, Pease CM. 1996. Science and management of Rocky mountain grizzly bears. Conserv Biol 10(4): 1013-1025.

McEwen BS. 1998. Stress, adaptation, and disease. Allostasis and allostatic load. Ann N Y Acad Sci 840: 33-44.

McEwen BS. 2000. The neurobiology of stress: from serendipity to clinical relevance. Brain Res 886(1-2): 172-189.

McEwen BS, Seeman T. 1999. Protective and damaging effects of mediators of stress. Elabourating and testing the concepts of allostasis and allostatic load. Ann N Y Acad Sci 896: 30-47.

McEwen BS, Wingfield JC. 2003. The concept of allostasis in biology and biomedicine. Horm Behav 43(1): 2-15.

McEwen BS, Wingfield JC. 2010. What is in a name? Integrating homeostasis, allostasis and stress. Horm Behav 57(2): 105-11.

McGarigal K, Cushman S, Stafford S. 2000. Multivariate statistics for wildlife and ecology research. New York: Springer-Verlag.

McLellan BN, Hovey FW, Mace RD, Woods JG, Carney DW, Gibeau ML, Wakkinen WL, Kasworm WF. 1999. Rates and causes of grizzly bear mortality in the interior mountains

of British Columbia, Alberta, Montana, Washington, and Idaho. J Wildl Manage 63(3): 911-920.

McLoughlin PD, Boyce MS, Coulson T, Clutton-Brock T. 2006. Lifetime reproductive success and density-dependent, multi-variable resource selection. Proc Biol Sci 273(1593): 1449-54.

Mege RM, Gavard J, Lambert M. 2006. Regulation of cell-cell junctions by the cytoskeleton. Curr Opin Cell Biol 18(5): 541-8.

Merino S, Moreno J, Tomas G, Martinez J, Morales J, Martinez-de la Puente J, Osorno JL. 2006. Effects of parental effort on blood stress protein HSP60 and immunoglobulins in female blue tits: a brood size manipulation experiment. J Anim Ecol 75(5): 1147-53.

Millennium Ecosystem Assessment. 2005. Ecosystems and human well-being: biodiversity synthesis. Washington, DC: World Resources Institute.

Moens LN, van der Ven K, Van Remortel P, Del-Favero J, De Coen WM. 2006. Expression profiling of endocrine-disrupting compounds using a customized Cyprinus carpio cDNA microarray. Toxicol Sci 93(2): 298-310.

Moll R, Divo M, Langbein L. 2008. The human keratins: biology and pathology. Histochem Cell Biol 129(6): 705-33.

Momoda TS, Schwindt AR, Feist GW, Gerwick L, Bayne CJ, Schreck CB. 2007. Gene expression in the liver of rainbow trout, Oncorhynchus mykiss, during the stress response. Comp Biochem Physiol 2: 303-315.

Monastyrskaya K, Babiychuk EB, Draeger A. 2009. The annexins: spatial and temporal coordination of signaling events during cellular stress. Cell Mol Life Sci 66(16): 2623-42.

Monfort SL, Brown JL, Wildt DE. 1993. Episodic and seasonal rhythms of cortisol secretion in male Eld's deer (*Cervus eldi thamin*). J Endocrinol 138: 41-49.

Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E and others. 2003. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet 34(3): 267-73.

Mowat G, Heard DC, Seip DR, Poole KG, Stenhouse G, Paetkau DW. 2005. Grizzly Ursus arctos and black bear U. americanus densities in the interior mountains of North America. Wildl Biol 11: 31-48.

Mujumdar RB, Ernst LA, Mujumdar SR, Lewis CJ, Waggoner AS. 1993. Cyanine dye labeling reagents: sulfoindocyanine succinimidyl esters. Bioconjug Chem 4(2): 105-11.

Munson L, Terio KA, Worley M, Jago M, Bagot-Smith A, Marker L. 2005. Extrinsic factors significantly affect patterns of disease in free-ranging and captive cheetah (*Acinonyx jubatus*) populations. J Wildl Dis 41(3): 542-8.

Nakano Y. 2004. Stress-induced modulation of skin immune function: two types of antigen-presenting cells in the epidermis are differentially regulated by chronic stress. Br J Dermatol 151(1): 50-64.

Naves J, Wiegand T, Revilla E, Delibes M. 2003. Endangered species constrained by natural and human factors: the case of brown bears in Northern Spain. Conserv Biol 17(5): 1276-1289.

Nielsen S, editor. 2010. Grizzly bear habitat productivity models for the Yellowhead, Swan Hills, Grande Cache and Chinchaga population units of Alberta. Hinton, Alberta. 58-81 p.

Nielsen SE, Boyce MS, Stenhouse GB. 2004a. Grizzly bears and forestry I. Selection of clearcuts by grizzly bears in west-central Alberta, Canada. For Ecol Mange 199: 51-65.

Nielsen SE, Cranston J, Stenhouse GB. 2009. Identification of priority areas for grizzly bear conservation and recovery in Alberta, Canada. J Cons Plan 5: 38-60.

Nielsen SE, Herrero S, Boyce MS, Mace RD, Benn B, Gibeau ML, Jevons S. 2004b. Modeling the spatial distribution of human-caused grizzly bear mortalities in the Central Rockies ecosystem of Canada. Biol Conserv 120(1): 101-113.

Nielsen SE, Johnson CJ, Heard DC, Boyce MS. 2005. Can models of presence-absence be used to scale abundance? Two case studies considering extremes in life history. Ecogeography 28(2): 197-208.

Nielsen SE, McDermid GJ, Stenhouse GB, Boyce MS. 2010. Dynamic wildlife habitat models: seasonal foods and mortality risk predict occupancy-abundance and habitat selection in grizzly bears. Biol Conserv 143: 1623-1634.

Nielsen SE, Munro RHM, Bainbridge EL, Stenhouse GB, Boyce MS. 2004c. Grizzly bears and forestry II. Distribution of grizzly bear foods in clearcuts of west-central Alberta, Canada. For Ecol Mange 199: 67-82.

Nielsen SE, Stenhouse GB, Beyer HL, Huettmann F, Boyce MS. 2008. Can natural disturbance-

based forestry rescue a declining population of grizzly bears? Biol Cons 141: 2193-2207.

Nielsen SE, Stenhouse GB, Boyce MS. 2006. A habitat-based framework for grizzly bear conservation in Alberta. Biol Conserv 130: 217-229.

Olliff T, Legg K, Kaeding B, editors. 1999. Effects of winter recreation on wildlife of the Greater Yellowstone Area: a literture review and assessment. Yellowstone National Park, Wyoming. 315 p.

Omsjoe EH, Stien A, Irvine J, Albon SD, Dahl E, Thoresen SI, Rustad E, Ropstad E. 2009. Evaluating capture stress and its effects on reproductive success in Svalbard reindeer. Can J Zool 87(1): 73-85.

Panjwani NN, Popova L, Srivastava PK. 2002. Heat shock proteins gp96 and hsp70 activate the release of nitric oxide by APCs. J Immunol 168(6): 2997-3003.

Pasitschniak-Arts M. 1993. Ursus arctos. Mamm Spec 439: 1-10.

Paus R. 1991. Does prolactin play a role in skin biology and pathology? Med Hypotheses 36(1):33-42.

Paus R, Theoharides TC, Arck PC. 2006. Neuroimmunoendocrine circuitry of the 'brain-skin connection'. Trends Immunol 27(1): 32-9.

Pavlidis P, Qin J, Arango V, Mann JJ, Sibille E. 2004. Using the gene ontology for microarray data mining: a comparison of methods and application to age effects in human prefrontal cortex. Neurochem Res 29(6): 1213-22.

Pavlovic S, Daniltchenko M, Tobin DJ, Hagen E, Hunt SP, Klapp BF, Arck PC, Peters EM. 2008. Further exploring the brain-skin connection: stress worsens dermatitis via substance P-dependent neurogenic inflammation in mice. J Invest Dermatol 128(2): 434-46.

Perdew GH. 1988. Association of the Ah receptor with the 90-kDa heat shock protein. J Biol Chem 263(27): 13802-5.

Pockley AG. 2003. Heat shock proteins as regulators of the immune response. Lancet 362(9382): 469-76.

Pockley AG, Muthana M, Calderwood SK. 2008. The dual immunoregulatory roles of stress proteins. Trends Biochem Sci 33(2): 71-9.

Proctor M, Paetkau D. 2004. A genetic-based spatial analysis of grizzly bears in Alberta. Commissioned by: Alberta Sustainable Resource Development Fish and Wildlife

Division. 1-27 p.

Proctor MF, McLellan BN, Strobeck C, Barclay RM. 2005. Genetic analysis reveals demographic fragmentation of grizzly bears yielding vulnerably small populations. Proc Biol Sci 272(1579): 2409-16.

Quinones QJ, de Ridder GG, Pizzo SV. 2008. GRP78: a chaperone with diverse roles beyond the endoplasmic reticulum. Histol Histopathol 23(11): 1409-16.

Raine RM, Riddell RN. 1991. Grizzly bear research in Yoho and Kootenay National Parks. Calgary, AB: Canadian Parks Service Report.

Reed SE, Merenlender AM. 2008. Quiet, nonconsumptive recreation reduces protected area effectiveness. Conserv Lett 1: 146-154.

Reich A, Wojcik-Maciejewicz A, Slominski AT. 2010. Stress and the skin. G Ital Dermatol Venereol 145(2): 213-9.

Rhee SG, Chae HZ, Kim K. 2005. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. Free Radic Biol Med 38(12): 1543-52.

Ricklefs RE, Wikelski M. 2002. The physiology/life-history nexus. Trend Ecol Evol 17(10): 462-468.

Ritossa F. 1962. A new puffing pattern induced by temperature shock and DNP in Drosphila. Experientia 18: 571-573.

Roca AL, Georgiadis N, Pecon-Slattery J, O'Brien SJ. 2001. Genetic evidence for two species of elephant in Africa. Science 293: 1473-1477.

Rode KD, Farley SD, Robbins CT. 2006. Sexual dimorphism, reproductive strategy, and human activities determine resource use by brown bears. Ecology 87: 2636-2646.

Rode KD, Robbins CT, Shipley LA. 2001. Constraints on herbivory by grizzly bears. Oecologia 128: 62-71.

Rodrigo MC, Martin DS, Redetzke RA, Eyster KM. 2002. A method for the extraction of high-quality RNA and protein from single small samples of arteries and veins preserved in RNAlater. J Pharmacol Toxicol Methods 47(2): 87-92.

Roever CL, Boyce MS, Stenhouse GB. 2010. Grizzly bear movements relative to roads: application of step selection functions. Ecogeography 33: 1113-1122.

Rogalla T, Ehrnsperger M, Preville X, Kotlyarov A, Lutsch G, Ducasse C, Paul C, Wieske M,

Arrigo AP, Buchner J and others. 1999. Regulation of Hsp27 oligomerization, chaperone function, and protective activity against oxidative stress/tumor necrosis factor alpha by phosphorylation. J Biol Chem 274(27): 18947-56.

Romero LM. 2004. Physiological stress in ecology: lessons from biomedical research. TRENDS Ecol Evol 19(5): 249-255.

Romero LM, Dickens MJ, Cyr NE. 2009. The Reactive Scope Model - a new model integrating homeostasis, allostasis, and stress. Horm Behav 55(3): 375-89.

Ross PI. 2002. COSEWIC assessment and update status report on the grizzly bear Ursus arctos in Canada. 1-91 p.

Sanders BM. 1993. Stress proteins in aquatic organisms: an environmental perspective. Crit Rev Toxicol 23(1): 49-75.

Sapolsky RM. 1992. Neuroendocrinology of the stress-response. In: Becker JB, Breedlove SM, Crews D, editors. Behavioural Endocrinology. Cambridge, MA: MIT Press. p 287-324.

Sapolsky RM, Romero LM, Munck AU. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev 21(1): 55-89.

Scheibel T, Buchner J. 1998. The Hsp90 complex--a super-chaperone machine as a novel drug target. Biochem Pharmacol 56(6): 675-82.

Schlaepfer DD, Haigler HT. 1990. Expression of annexins as a function of cellular growth state. J Cell Biol 111(1): 229-38.

Schmidt MV, Sterlemann V, Muller MB. 2008. Chronic stress and individual vulnerability. Ann N Y Acad Sci 1148: 174-83.

Schneider JE. 2004. Energy balance and reproduction. Physiol Behav 81(2): 289-317.

Schneider RR. 2002. Alternative futures: Alberta's boreal forest at the crossroads. Research TFoANatACfB, editor. Edmonton, Alberta. 152 p.

Schwartz CC, Miller SD, Haroldson MA. 2003. Grizzly Bear. In: Feldhamer GA, Thompson BC, Chapman JA, editors. Wild Mammals of North America: Biology, Management, and Conservation. second ed. Baltimore: The John Hopkins University Press. p 556-586.

Schweitzer B, Roberts S, Grimwade B, Shao W, Wang M, Fu Q, Shu Q, Laroche I, Zhou Z, Tchernev VT and others. 2002. Multiplexed protein profiling on microarrays by rolling-circle amplification. Nat Biotechnol 20(4): 359-65.

Seeman T, Epel E, Gruenewald T, Karlamangla A, McEwen BS. 2010. Socio-economic differentials in peripheral biology: cumulative allostatic load. Ann N Y Acad Sci 1186: 223-39.

Seeman TE, Crimmins E, Huang MH, Singer B, Bucur A, Gruenewald T, Berkman LF, Reuben DB. 2004. Cumulative biological risk and socio-economic differences in mortality: MacArthur studies of successful aging. Soc Sci Med 58(10): 1985-97.

Seeman TE, Singer BH, Rowe JW, Horwitz RI, McEwen BS. 1997. Price of adaptation--allostatic load and its health consequences. MacArthur studies of successful aging. Arch Intern Med 157(19): 2259-68.

Selye H. 1936. A syndrome produced by diverse nocuous agents. Nature 138: 32.

Seplaki CL, Goldman N, Glei D, Weinstein M. 2005. A comparative analysis of measurement approaches for physiological dysregulation in an older population. Exp Gerontol 40(5): 438-49.

Shamaei-Tousi A, Steptoe A, O'Donnell K, Palmen J, Stephens JW, Hurel SJ, Marmot M, Homer K, D'Aiuto F, Coates AR and others. 2007. Plasma heat shock protein 60 and cardiovascular disease risk: the role of psychosocial, genetic, and biological factors. Cell Stress Chaperones 12(4): 384-92.

Shaner L, Morano KA. 2007. All in the family: atypical Hsp70 chaperones are conserved modulators of Hsp70 activity. Cell Stress Chaperones 12(1): 1-8.

Shaw MM, Riederer BM. 2003. Sample preparation for two-dimensional gel electrophoresis. Proteomics 3(8): 1408-17.

Sirover MA. 1997. Role of the glycolytic protein, glyceraldehyde-3-phosphate dehydrogenase, in normal cell function and in cell pathology. J Cell Biochem 66(2): 133-40.

Slominski A, Pisarchik A, Tobin DJ, Mazurkiewicz JE, Wortsman J. 2004. Differential expression of a cutaneous corticotropin-releasing hormone system. Endocrinology 145(2): 941-50.

Slominski A, Wortsman J. 2000. Neuroendocrinology of the skin. Endocr Rev 21(5): 457-87.

Slominski A, Wortsman J, Luger T, Paus R, Solomon S. 2000. Corticotropin releasing hormone and proopiomelanocortin involvement in the cutaneous response to stress. Physiol Rev 80(3): 979-1020.

Slominski A, Wortsman J, Paus R, Elias PM, Tobin DJ, Feingold KR. 2008. Skin as an

endocrine organ: implications for its function. Drug Discov Today Dis Mech 5(2): 137-144.

Slominski A, Wortsman J, Pisarchik A, Zbytek B, Linton EA, Mazurkiewicz JE, Wei ET. 2001. Cutaneous expression of corticotropin-releasing hormone (CRH), urocortin, and CRH receptors. Faseb J 15(10): 1678-93.

Slominski A, Wortsman J, Tuckey RC, Paus R. 2007. Differential expression of HPA axis homolog in the skin. Mol Cell Endocrinol 265-266: 143-9.

Southern SO, Allen A, Keller N. 2002. Molecular signature of physiolgical stress in dolphins based on protein expression profiling of skin. La Jolla: Southwestern Fisheries Science Center. Report nr LJ-02-27. 31 p.

Spellerberg IF. 1998. Ecological effects of roads and traffic: a literature review. Global Ecol Biogeogr 7: 317-333.

Spencer RL, Kalman BA, Dhabhar FS. 2001. Role of endogenous glucocorticoids in immune system function: regulation and counterregulation. In: McEwen BS, Goodman HM, editors. Handbook of physiology. New York: Oxford university press. p 381-423.

Spisak S, Guttman A. 2009. Biomedical applications of protein microarrays. Curr Med Chem 16(22): 2806-15.

St. Aubin DJ, Austin TP, Geraci JR. 1979. Effects of handling stress on plasma enzymes in harp seals, Phoca groenlandica. J Wildl Dis 15(4): 569-572.

Steer SA, Corbett JA. 2003. The role and regulation of COX-2 during viral infection. Viral Immunol 16(4): 447-60.

Stenhouse GB, Graham K, editors. 2008. Foothills research institute grizzly bear program 2007 annual report. Hinton, Alberta. 204 p.

Stenhouse GB, Munro R, Graham K. 2003. Foothills Model Forest Grizzly Bear Research Program 2002 annual report (year 4 of a 5 year study). Hinton, Alberta. 162 p.

Sterlemann V, Ganea K, Liebl C, Harbich D, Alam S, Holsboer F, Muller MB, Schmidt MV. 2008. Long-term behavioural and neuroendocrine alterations following chronic social stress in mice: implications for stress-related disorders. Horm Behav 53(2): 386-94.

Storer TI, Trevis LP. 1955. California grizzly: University of Nebraska Press.

Struhsaker TT. 1976. A further decline in numbers of Amboseli: vervet monkeys. Biotropica 8: 211-214.

Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES and others. 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 102: 15545-15550.

Tang A, Amagai M, Granger LG, Stanley JR, Udey MC. 1993. Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin. Nature 361(6407): 82-5.

Targher G. 2010. Elevated serum gamma-glutamyltransferase activity is associated with increased risk of mortality, incident type 2 diabetes, cardiovascular events, chronic kidney disease and cancer - a narrative review. Clin Chem Lab Med 48(2): 147-57.

Tarze A, Deniaud A, Le Bras M, Maillier E, Molle D, Larochette N, Zamzami N, Jan G, Kroemer G, Brenner C. 2007. GAPDH, a novel regulator of the pro-apoptotic mitochondrial membrane permeabilization. Oncogene 26(18): 2606-20.

Templin MF, Stoll D, Schwenk JM, Potz O, Kramer S, Joos TO. 2003. Protein microarrays: promising tools for proteomic research. Proteomics 3(11): 2155-66.

Terio KA, Marker L, Munson L. 2004. Evidence for chronic stress in captive but not free-ranging cheetahs (*Acinonyx jubatus*) based on adrenal morphology and function. J Wildl Dis 40(2): 259-66.

Tilbrook AJ, Clarke IJ. 2006. Neuroendocrine mechanisms of innate states of attenuated responsiveness of the hypothalamo-pituitary adrenal axis to stress. Front Neuroendocrinol 27(3): 285-307.

Titus JR, VanDruff LW. 1981. Response of the common loon to recreational pressure in the boundary waters canoe area, Northeastern Minnesota. Wildl Monogr 79: 1-59.

Tomei F, Ciarrocca M, Cherubini E, Rosati MV, Monti C, Capozzella A, Tomei G. 2006. Prolactin levels in workers exposed to chemical, physical and psycho-social urban stressors. J Occup Health 48(4): 253-60.

Topal A, Gul NY, Yanik K. 2010. Effect of capture method on hematological and serum biochemical values of red deer (*Cervus elaphus*) in Turkey. J Anim Vet Adv 9(8): 1227-1231.

Tovar H, Navarrete F, Rodriguez L, Skewes O, Castro FO. 2008. Cold storage of biopsies from wild endangered native Chilean species in field conditions and subsequent isolation of primary culture cell lines. In Vitro Cell Dev Biol Anim 44(8-9): 309-20.

Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76(9): 4350-4.

Tripp KM, Verstegen JP, Deutsch CJ, Bonde RK, Wit MD, Manire CA, Gaspard J, Harr KE. 2010. Evaluation of adrenocortical function in Florida manatees (*Trichechus manatus latirostris*). Zoo Biol.

Tsigos C, Chrousos GP. 2002. Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. J Psychosom Res 53(4): 865-71.

van Empel VP, Bertrand AT, van der Nagel R, Kostin S, Doevendans PA, Crijns HJ, de Wit E, Sluiter W, Ackerman SL, De Windt LJ. 2005. Downregulation of apoptosis-inducing factor in harlequin mutant mice sensitizes the myocardium to oxidative stress-related cell death and pressure overload-induced decompensation. Circ Res 96(12): e92-e101.

Vanden Berghe T, Kalai M, van Loo G, Declercq W, Vandenabeele P. 2003. Disruption of HSP90 function reverts tumor necrosis factor-induced necrosis to apoptosis. J Biol Chem 278(8): 5622-9.

Volpi S, Rabadan-Diehl C, Aguilera G. 2004. Vasopressinergic regulation of the hypothalamic pituitary adrenal axis and stress adaptation. Stress 7(2): 75-83.

von der Ohe CG, Wasser SK, Hunt KE, Servheen C. 2004. Factors associated with fecal glucocorticoids in Alaskan brown bears (*Ursus arctos horribilis*). Physiol Biochem Zool 77(2): 313-20.

Walter G, Bussow K, Lueking A, Glokler J. 2002. High-throughput protein arrays: prospects for molecular diagnostics. Trends Mol Med 8(6): 250-3.

Wandinger SK, Richter K, Buchner J. 2008. The Hsp90 chaperone machinery. J Biol Chem 283(27): 18473-7.

Wasser SK, Bevis K, King G, Hanson E. 1997. Noninvasive physiological measures of disturbance in the northern spotted owl. Conserv Biol 11(4): 1019-1022.

Wasser SK, Davenport B, Ramage ER, Hunt KE, Parker M, Clarke C, Stenhouse GB. 2004. Scat detection dogs in wildlife research and management: application to grizzly and black bears in the Yellowhead Ecosystem, Alberta, Canada. Can. J. Zool. 82: 475-492.

Wastling JM, Xia D, Sohal A, Chaussepied M, Pain A, Langsley G. 2009. Proteomes and transcriptomes of the Apicomplexa--where's the message? Int J Parasitol 39(2): 135-43.

Weaver JL, Paquet PC, Ruggiero LF. 1996. Resilience and conservation of large carnivores in the Rocky Mountains. Conserv Biol 10(4): 964-976.

Weber DK, Danielson K, Wright S, Foley JE. 2002. Hematology and serum biochemistry values of dusky-footed wood rat (*Neotoma fuscipes*). J Wildl Dis 38(3): 576-82.

Webster JI, Tonelli L, Sternberg EM. 2002. Neuroendocrine regulation of immunity. Annu Rev Immunol 20: 125-63.

Webster Marketon JI, Glaser R. 2008. Stress hormones and immune function. Cell Immunol 252(1-2): 16-26.

Welch WJ. 1993. Heat shock proteins functioning as molecular chaperones: their roles in normal and stressed cells. Philos Trans R Soc Lond B Biol Sci 339(1289): 327-33.

Wikelski M, Cooke SJ. 2006. Conservation physiology. TRENDS Ecol Evol 21(2): 38-36.

Willoughby DA, Moore AR, Colville-Nash PR, Gilroy D. 2000. Resolution of inflammation. Int J Immunopharmacol 22(12): 1131-5.

Wilson DS, Nock S. 2003. Recent developments in protein microarray technology. Angew Chem Int Ed Engl 42(5): 494-500.

Wingfield JC. 2005. The concept of allostasis: coping with a capricious environment. J Mammal 86(2): 248-254.

Wingfield JC, Romero LM. 2001. Adrenocortical responses to stress and their modulation in free-living vertebrates. In: McEwen BS, Goodman HM, editors. Handbook of physiology. New York: Oxford university press. p 211-234.

Wobeser GA. 2006. Essentials of disease in wild animals. Ames, Iowa: Blackwell.

Wonsey DR, Zeller KI, Dang CV. 2002. The c-Myc target gene PRDX3 is required for mitochondrial homeostasis and neoplastic transformation. Proc Natl Acad Sci U S A 99(10): 6649-54.

Xanthoudakis S, Nicholson DW. 2000. Heat-shock proteins as death determinants. Nat Cell Biol 2(9): E163-5.

Zar JH. 1999. Biostatistical Analysis. Singapore: Pearson Education.

Zaric J, Ruegg C. 2005. Integrin-mediated adhesion and soluble ligand binding stabilize COX-2 protein levels in endothelial cells by inducing expression and preventing degradation. J Biol Chem 280(2): 1077-85.

Zelko IN, Mariani TJ, Folz RJ. 2002. Superoxide dismutase multigene family: a comparison of

the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radic Biol Med 33(3): 337-49.

Zhang LH, Zhang X. 2010. Roles of GRP78 in Physiology and Cancer. J Cell Biochem 110(6):1299-305.

Zhivotovsky B, Orrenius S. 2005. Caspase-2 function in response to DNA damage. Biochem Biophys Res Commun 331(3): 859-67.

Zhou H, Bouwman K, Schotanus M, Verweij C, Marrero JA, Dillon D, Costa J, Lizardi P, Haab BB. 2004. Two-color, rolling-circle amplification on antibody microarrays for sensitive, multiplexed serum-protein measurements. Genome Biol 5(4): R28.

Zugel U, Kaufmann SH. 1999. Role of heat shock proteins in protection from and pathogenesis of infectious diseases. Clin Microbiol Rev 12(1): 19-39.

## Appendix 1

## Validation of Antibodies

**Table A1.1** Validation of antibodies selected from the Hypromatrix commercial array by western blotting. Also noted is whether the antibody was selected for the custom bear microarray.

| Antibody | Catalog No. | Commercial Array | Validated by Western blotting | Selected for bear specific array? |
|---|---|---|---|---|
| eNOS (endothelial nitric oxide synthase) | HM1253 | Hypromatrix | No | No |
| ISGF-3 γ (Interferon-stimulated transcription factor 3γ) | HM1202 | Hypromatrix | No | No |
| Lck (leukocyte-specific protein tyrosine kinase) | HM1346 | Hypromatrix | No | No |
| L-selectin (leukocyte) | HM1329 | Hypromatrix | No | No |
| MGMT (O 6 -methylguanine-DNA methyltransferase) | HM1227 | Hypromatrix | No | No |
| nip2 (Nek2-interacting protein 2) | HM1251 | Hypromatrix | No | No |
| PARP (poly (ADP-ribose) transferase) | HM1277 | Hypromatrix | No | No |
| Pax5 (Paired box gene 5) | HM1278 | Hypromatrix | No | No |
| Phospholipase D | HM1284 | Hypromatrix | No | No |
| p-selectin (platelet)(ELMA-1) | HM1330 | Hypromatrix | No | No |
| p-Stat3 (phosphorylated-signal transducer and activator of transcription 3) | HM1356 | Hypromatrix | No | No |
| RACK1 (receptor for activated C kinase) | HM1301 | Hypromatrix | No | No |
| Rad51 (DNA repair protein) | HM1302 | Hypromatrix | No | No |
| RAIDD (RIP associated ICH-1/Ced-3 homologous protein with a death domain) | HM1305 | Hypromatrix | No | No |
| Ran BP-1 (Ran/TC4-binding protein) | HM1412 | Hypromatrix | No | No |
| Rel B | HM1320 | Hypromatrix | No | No |
| Sam68 (Src-associated in mitosis 68 kDa protein) | HM1327 | Hypromatrix | No | No |
| SHC ((Src homology 2 containing) transforming protein) | HM1331 | Hypromatrix | No | No |
| Sik (Src-related intestinal kinase) | HM1333 | Hypromatrix | No | No |
| Stat2 (signal transducer and activator of transcription) | HM1354 | Hypromatrix | No | No |
| Stat4 | HM1357 | Hypromatrix | No | No |
| TRAF2 (TNF receptor associated factor) | HM1378 | Hypromatrix | No | No |
| TRAF3 | HM1379 | Hypromatrix | No | No |
| TRAF4 | HM1380 | Hypromatrix | No | No |
| XRCC4 (x-ray repair cross-complementing protein-4) | HM1397 | Hypromatrix | No | No |

**Table A1.2** Validation of antibodies selected from the Sigma commercial array by western blotting. Also noted is whether the antibody was selected for the custom bear microarray.

| Antibody | Catalog No. | Commercial Array | Validated by Western blotting | Selected for bear specific array? |
|---|---|---|---|---|
| AOP-1 (Antioxidant-like Protein 1) | A7674 | Sigma | No | No |
| Caspase 3 | C9598 | Sigma | No | No |
| Caspase 3 (active) | C8487 | Sigma | No | No |
| Caspase 6 | C7599 | Sigma | **Yes** | **Yes** |
| Estrogen receptor | E0521 | Sigma | No | No |
| Hsp70 (i + c) (heat shock protein 70 inducible and constitutive) | H5147 | Sigma | No | No |
| iNOS (Nitric Oxide Synthase, inducible) | N7782 | Sigma | **Yes** | **Yes** |
| iNOS | N9657 | Sigma | No | No |
| MAP Kinase (Mitogen-activated protein kinase) (ERK 1, 351-368) | M7927 | Sigma | **Yes** | No |
| MAP kinase p38 | M0800 | Sigma | **Yes** | No |
| MAP Kinase-activated protein kinase 2 | M3550 | Sigma | No | No |
| Nerve Growth Factor Receptor | N5408 | Sigma | No | No |
| PKBα/Akt1 (Protein kinase B alpha) | P1601 | Sigma | **Yes** | No |
| PRDX3 (Peroxiredoxin 3) | P1247 | Sigma | **Yes** | No |

**Table A1.3** Validation of antibodies selected from the Spring Bioscience commercial array by western blotting. Also noted is whether the antibody was selected for the custom bear microarray.

| Antibody | Catalog No. | Commercial Array | Validated by Western blotting | Selected for bear specific array? |
|---|---|---|---|---|
| 6-Histidine | E6984 | Spring Bioscience | No | No |
| CD2 (Cluster of differentiation 2) | E3044 | Spring Bioscience | No | No |
| CD20 | E2564 | Spring Bioscience | No | No |
| CD5 | M3194 | Spring Bioscience | No | No |
| CD74 | E131 | Spring Bioscience | No | No |
| Collagen II | E5634 | Spring Bioscience | No | No |
| Cullin-1 | E3794 | Spring Bioscience | No | No |
| GCDFP-15 (Gross Cystic Disease Fluid Protein15) | E1684 | Spring Bioscience | No | No |
| Hepatic Nuclear Factor 3B | E1299 | Spring Bioscience | No | No |
| Keratin 19 | E5924 | Spring Bioscience | No | No |
| MAP2a,b | E6114 | Spring Bioscience | No | No |
| MMP-10 (Matrix Metalloproteinase-10) | E6734 | Spring Bioscience | No | No |
| MPA (Medroxyprogesterone Acetate) | E1047 | Spring Bioscience | No | No |
| Myogenin | E1964 | Spring Bioscience | No | No |
| Myoglobin | E2994 | Spring Bioscience | **Yes** | No |
| p57 | E6354 | Spring Bioscience | No | No |
| Plasma Cell Marker | E6374 | Spring Bioscience | No | No |
| RNP (Ribonucleoprotein) | E6424 | Spring Bioscience | No | No |
| SIRP alpha (Signal Regulatory Protein) | E6454 | Spring Bioscience | No | No |

**Appendix 2**

Stress protein differential score was calculated for each protein category by assigning a point to each bear for each protein expression at or above the 90$^{th}$ percentile or at or below the 10$^{th}$ percentile in expression for that protein and summing the points for each bear by protein category.

Model selection for HPA differential score without the environmental variables suggested that the HPA differential score varied by log hsp60 serum (F=5.8, p=0.02) and log hsp70 serum (F=6.3, p=0.01) (Adjusted $R^2$=0.07, n=108). Model selection for HPA differential score with the environmental variables suggested that HPA differential score varied by log hsp70 serum (F=3.5, p=0.07) and proportion protected (F=5.1, p=0.03) (Adjusted $R^2$=0.08, n=67).

Model selection for ACC differential score without the environmental variables suggested that ACC differential score varied by region (F=2.2, p=0.092), region*capture year interaction (F=2.2, p=0.092) and log hsp60 serum (F=5.4, p=0.02) (Adjusted $R^2$=0.02, n=108). Model selection for ACC differential score including the environmental variables suggested that ACC differential score varied by mean RSF (F=3.8, p=0.06) (Adjusted $R^2$=0.04, n=72).

Model selection for CS differential score without the environmental variables suggested that CS differential score varied by log hsp60 serum (F=6.1, p=0.02) and log hsp70 serum (F=3.4, p=0.07) (Adjusted $R^2$=0.06, n=108). Model selection for CS differential score with the environmental variables suggested that CS differential score varied by region (F=3.2, p=0.03), log hsp70 serum (F=3.3, p=0.08), proportion protected (F=9.4, p=0.003) and mean RSF (F=6.0, p=0.02) (Adjusted $R^2$=0.18, n=9-37). After controlling for hsp70 serum, proportion of habitat protected and mean RSF, bears from FMF core were found to have elevated CS differential score compared to those originating from North highway 16 (p=0.031) and Swan Hills (p=0.03) (Figure A2.1).

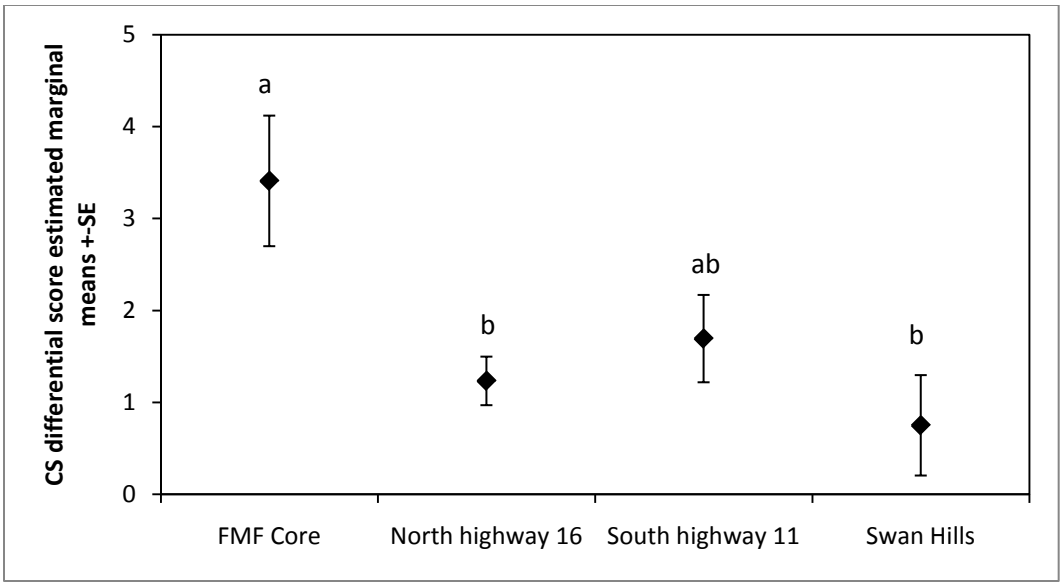


**Figure A2.1** Regional comparison of the cellular stress (CS) differential score modeled with environmental measures. Data points labeled with different letters are significantly different from each other, as determined by repeated measures ANOVA followed by Sidak test ($p<0.03$).

Model selection for OSI differential score without the environmental variables suggested that OSI differential score varied by log hsp60 serum ($F=7.2$, $p=0.01$) and log hsp70 serum ($F=7.8$, $p=0.01$) (Adjusted $R^2=0.09$, $n=107$). Model selection for OSI differential score with the environmental variables suggested that OSI differential score varied by log hsp70 serum ($F=5.8$, $p=0.02$), proportion protected ($F=6.0$, $p=0.02$) and mean RSF ($F=5.0$, $p=0.03$) (Adjusted $R^2=0.16$, $n=66$).

Model selection for protein differential score without the environmental variables suggested that protein differential score varied by log hsp60 serum ($F=7.5$, $p=0.01$) and log hsp70 serum ($F=6.4$, $p=0.01$) (Adjusted $R^2=0.09$, $n=107$). Model selection for protein differential score including the environmental variables suggested that protein differential score varied by log hsp70 serum ($F=5.2$, $p=0.03$), proportion protected ($F=5.5$, $p=0.02$) and mean RSF ($F=7.3$, $p=0.01$) (Adjusted $R^2=0.17$, $n=66$).

## Appendix 3

Stress protein index was calculated for each protein category by assigning a point to each bear for each protein expression at or above the 75$^{th}$ percentile in expression for that protein and summing the points for each bear by protein category.

Model selection for HPA index without the environmental variables suggested that HPA index varied by region (F=4.3, p=0.007), log length (cm) (F=3.3, p=0.07) and log hsp60 serum (F=7.7, p=0.007) (Adjusted $R^2$=0.15, n=100). After controlling for length and serum hsp60, bears from South highway 11 were found to have lower HPA index compared to those originating from North highway 16 (p=0.032) and Swan Hills (p=0.062) (Figure A3.1). Model selection for HPA index including the environmental variables suggested that HPA index varied by region (F=4.2, p=0.009), log length (cm) (F=4.1, p=0.05) and road density (F=3.2, p=0.08) (Adjusted $R^2$=0.16, n=65). After controlling for length and road density, bears from North highway 16 were found to have elevated HPA index compared to those originating from South highway 11 (p=0.005; data not shown due to low sample sizes).

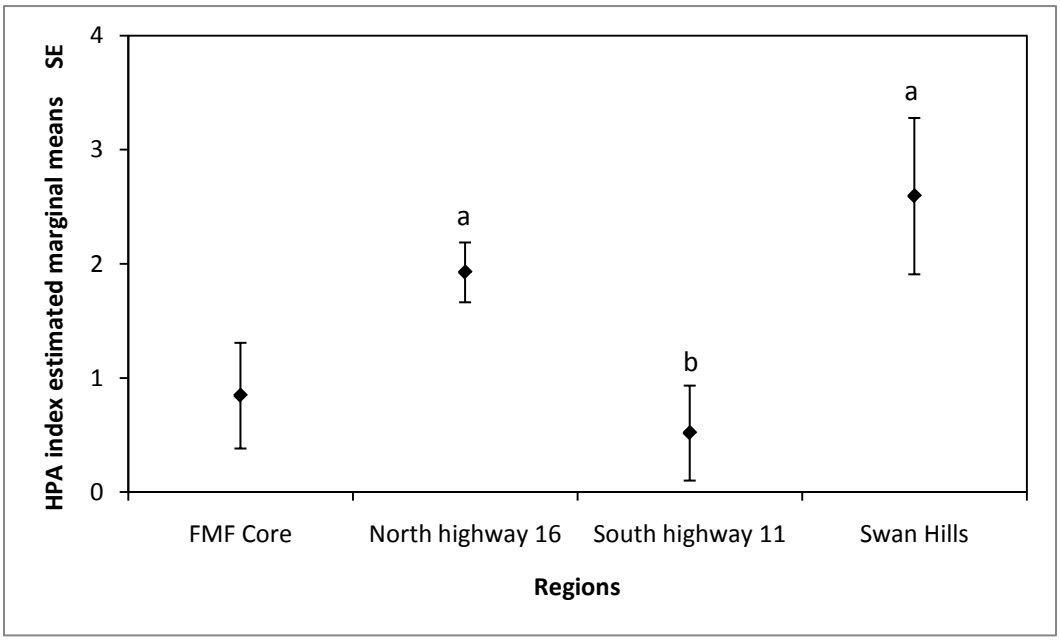


**Figure A3.1** Regional comparison of the hypothalamic-pituitary-adrenal axis (HPA) index modeled without environmental measures. Significant differences are labeled with letters (a>b), as determined by repeated measures ANOVA followed by Sidak test (p<0.065).

Model selection for ACC index excluding the environmental variables suggested that ACC index varied by region (F=6.9, p<0.001), capture year (F=6.4, p=0.01) and log serum hsp60 (ng/ml) (F=10.9, p=0.001) (Adjusted $R^2$=0.22, n=108). After controlling for capture year and serum hsp60, bears from South highway 11 were found to have depressed ACC index compared to those originating from North highway 16 (p=0.001) and Swan Hills (p=0.011) (Figure A3.2). Model selection for ACC index including the environmental variables suggested that ACC index varied by region (F=3.2, p=0.03), log serum hsp60 (ng/ml) (F=4.3, p=0.04) and road density (F=3.4, p=0.07) (Adjusted $R^2$=0.17, n=67). After controlling for serum hsp60 and road density, bears from North highway 16 were found to have elevated ACC index compared to those originating from South highway 11 (p=0.02; data not shown due to low sample sizes).

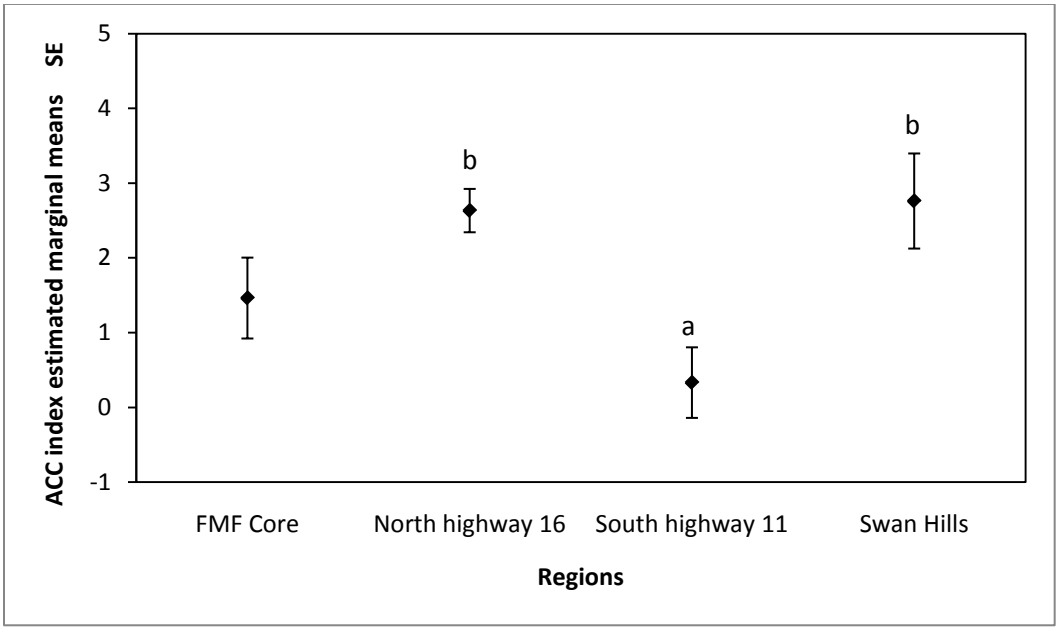


**Figure A3.2** Regional comparison of the apoptosis and cell cycle (ACC) index modeled without environmental measures. Significant differences are labeled with letters (a>b), as determined by repeated measures ANOVA followed by Sidak test (p<0.015).

Model selection for CS index excluding the environmental variables suggested that CS index varied by region (F=3.3, p=0.02) and log total serum cortisol (ng/ml) (F=3.0, p=0.09) (Adjusted $R^2$=0.08, n=109). After controlling for total serum cortisol, bears from Swan Hills were found to have elevated CS index compared to those originating FMF Core (p=0.03), North highway 16 (p=0.03) and South highway 11 (p=0.06) (Figure A3.3). The addition of the environmental variables did not improve the CS index model.

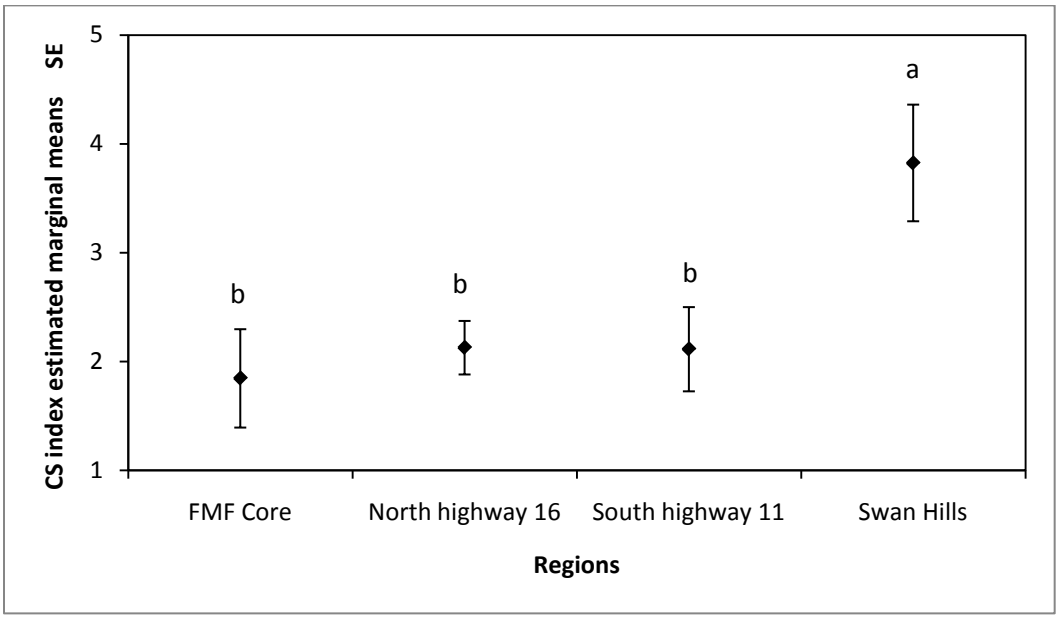


**Figure A3.3** Regional comparison of the cellular stress (CS) index modeled without environmental measures. Significant differences are labeled with letters (a>b), as determined by repeated measures ANOVA followed by Sidak test ($p<0.065$).

Model selection for OSI index excluding the environmental variables suggested that OSI index varied by region ($F=8.5$, $p<0.001$), capture year ($F=9.2$, $p=0.003$), log length (m) ($F=3.5$, $p=0.07$) and log serum hsp60 (ng/ml) ($F=3.9$, $p=0.05$) (Adjusted $R^2=0.28$, $n=100$). After controlling for capture year, length and serum hsp60, bears from Swan Hills were found to have elevated OSI index compared to those originating from FMF Core ($p=0.02$), South highway 11 ($p<0.001$), while bears from North highway 16 were found to have an elevated OSI index in comparison to bears originating from South highway 11 ($p=0.004$) (Figure A3.4). Model selection for OSI index including environmental variables suggested that OSI index varied by region ($F=7.2$, $p=0.002$), capture year ($F=6.1$, $p=0.02$), log length ($F=4.9$, $p=0.03$) and anthropogenic change ($F=4.8$, $p=0.03$) (Adjusted $R^2=0.22$, $n=49$). Controlling for capture year, length and anthropogenic change, bears from South highway 11 were found to have depressed OSI index compared to those originating from North highway 16 ($p=0.002$; data not shown due to low sample sizes).

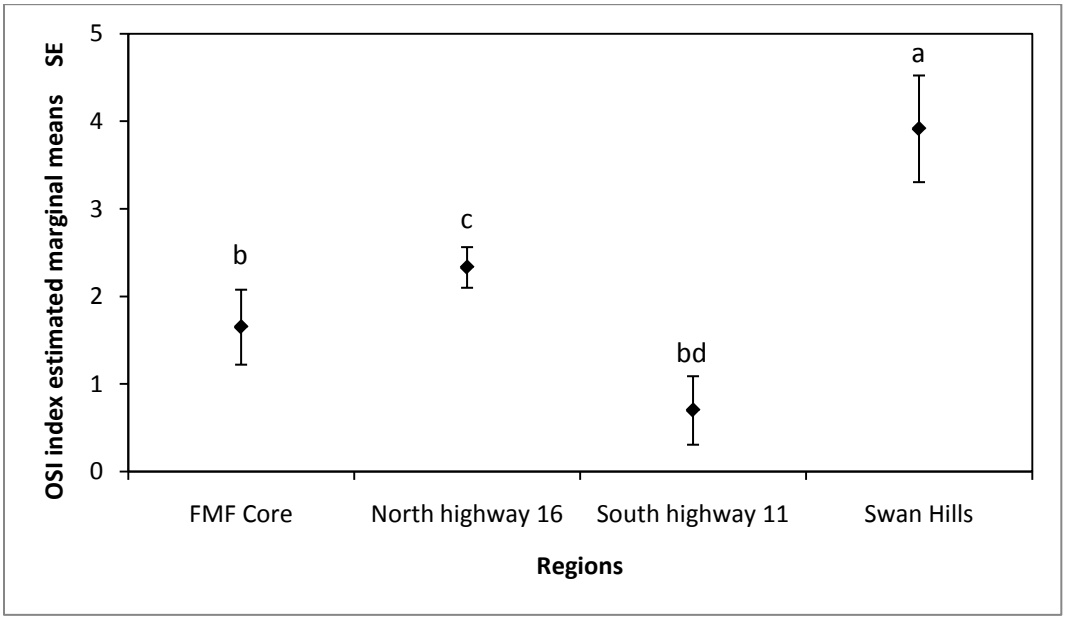


**Figure A3.4** Regional comparison of the oxidative stress and inflammation (OSI) index modeled without environmental measures. Significant differences are labeled with letters (a>b & c>d), as determined by repeated measures ANOVA followed by Sidak test ($p<0.025$).